Influence of sodium chloride on wine yeast physiology and fermentation performance

Stelios S. Logothetis

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I certify that this is the true and accurate version of the thesis approved by the examiners

Signed..... [redacted] **Date.....** 16/6/09

Director of Studies

To my father Sotiris, to my wife Athena and to my new born daughter Anastasia.

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ABSTRACT

This thesis concerns research into the influence of salt on physiology of the yeast, *Saccharomyces cerevisiae*. Specifically, the work focused on how sodium chloride affected the growth, viability and fermentation performance in industrial (winemaking) strains of this yeast in both laboratory-scale and industrial-scale experiments. Comparative fermentations were also conducted with selected non-*Saccharomyces* yeasts that are of relevance in enology. One of the main findings of the research presented involved the influence of salt "preconditioning" of yeasts which represents a method of pre-culturing cells in the presence of salt in an attempt to improve subsequent fermentation performance. Such an approach resulted in preconditioned yeasts having an improved capability to ferment high-sugar containing media with increased cell viability and with elevated levels of produced ethanol. Salt-preconditioning was most likely influencing the stress-tolerance of yeasts by inducing the synthesis of key metabolites such as trehalose and glycerol which act to improve cells' ability to withstand osmostress and ethanol toxicity. The industrial-scale trials using salt-preconditioned yeasts verified the benefit of the physiological engineering approach to practical winemaking fermentations. Benefits were also observed in a specialized fermentation system (WITY produced by the first letters of the words Wine, Immobilization, Tower, and Yeast) that utilized immobilized yeast. Overall, this research has demonstrated that a relatively simple method designed to physiologically adapt yeast cells – by salt-preconditioning – can have distinct advantages for alcohol fermentation processes.

Author Stelios Logothetis

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Address [redacted]

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1.0 INTRODUCTION

The influence of osmotic stress on cells, or "osmostress", has been widely studied. During the last decade, the application of hyper osmotic stress to microorganisms has been used to investigate secondary metabolic pathways and other cellular changes induced under such conditions. In yeast cells, osmostress triggers a series of biological responses in an effort to maintain cell viability and cell cycle progress. Many studies of osmostress in laboratory strains of *Saccharomyces cerevisiae* have focused on transcriptional activation, changes in protein synthesis, DNA damage and DNA recovery, gene expression and apoptotic phenomena.

The overall aim of my research work was to further our understanding of fermentation performance of industrial (winemaking) yeast strains under osmotic stress conditions and to elucidate the effect of stress on cell viability, metabolism and growth. Specifically, the research work focused on the evaluation of NaCl-induced stress responses on industrial wine yeast strains of *S. cerevisiae* (VIN 13, Vitilevure Chardonnay, Vitilevure SCM and Vitilevure KD) and on two non-*Saccharomyces* strains (*Kluyveromyces thermotolerans* and *Kluyveromyces marxianus*). The hypothesis was that osmotic stress conditions energized specific genes to enable yeast cells to survive under subsequent stressful conditions during fermentation. Experiments were designed by treating cells with different sodium chloride

concentrations (NaCl: 0% to 10% w/v) growing in defined media containing D-glucose and then evaluating the impact of this on yeast physiology. Secondly, industrial scale fermentations were performed with three wine yeast strains to evaluate pre-stressed (or "preconditioned") cells regarding their alcohol productivity, glycerol production and wine quality.

The introduction to this thesis starts with a general overview of winemaking and the different kind of stresses that impact on yeast during wine production. The thesis introduction then discusses yeast osmostress, with specific reference to NaCl-induced stress to *S. cerevisiae*.

1.1 Modern Wine Making

Modern wine making has significant demands concerning product consistency and quality and is driving winemakers to make the right choices at every stage of production; from grape harvest to wine bottling. Nowadays, the wine industry is truly global and contributes significantly in economic terms to many countries. Especially for the New World countries, research into fermentation and new yeast strains plays an important role in dictating wine quality and in governing wine production processes. In recent years, even when we have grapes from the same variety and from the same area the wine produced depends mainly on the yeast strain used. In summary, the choice of the strain will dictate the following:

- Fermentation performance
- Tolerance to different kinds of stresses
- Utilization of carbon sources
- Volatile compounds composition
- Yeast growth rate
- Alcohol productivity
- Higher alcohols composition
- Esters composition
- Carbonyl compounds
- Sulphur compounds

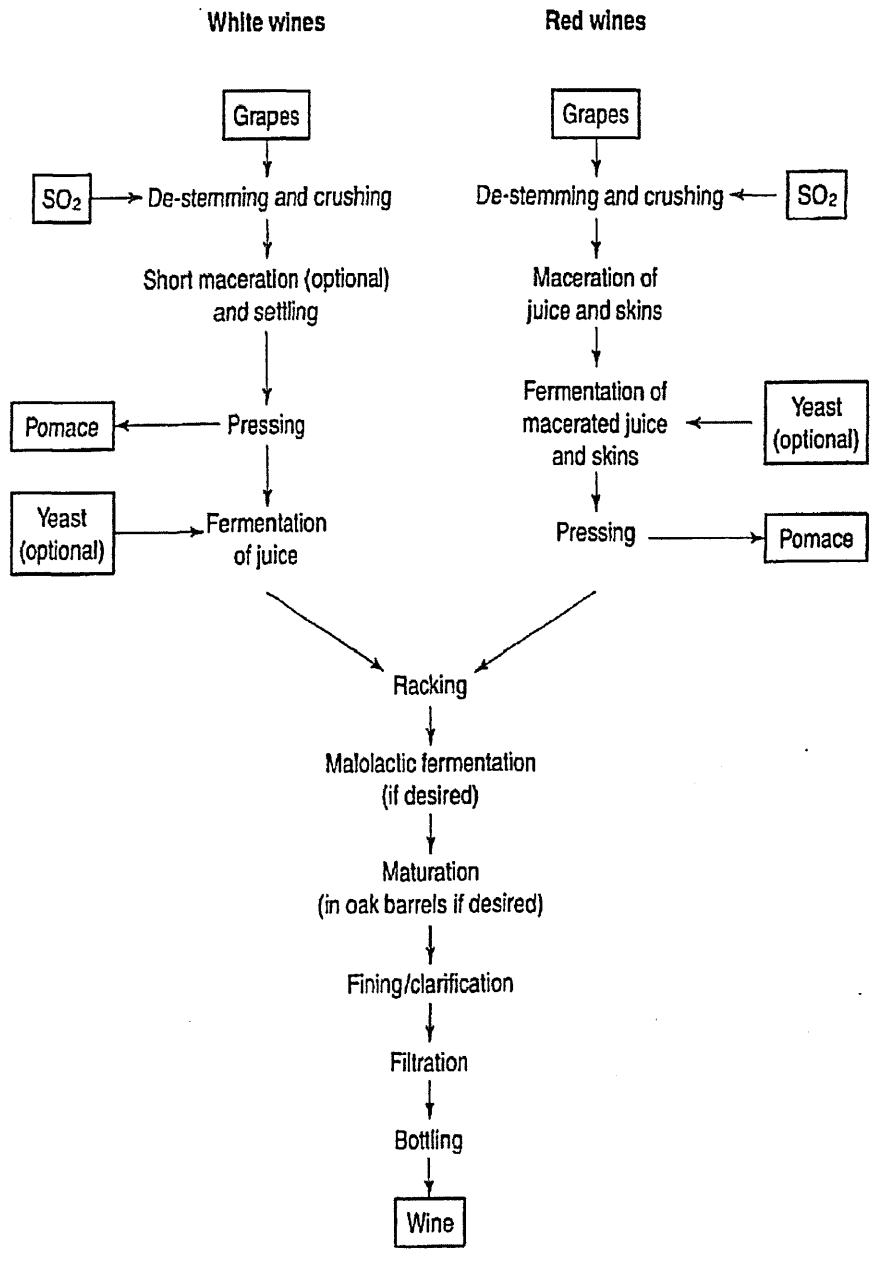


Figure 1 Summary of white and red winemaking (adapted from Walker, 1999)

Figure 1 describes the process of wine making in general. Some processes involve de-stemming and crushing, which are the same procedures for white and red wine making. Maceration is a process which begins as soon as the grapes skins are broken and exposed under low or high temperatures. Temperature is the guiding force and is very important for the breakdown and extraction of phenols and aromatic compounds during white winemaking and for the extraction of anthocyanins, flavour compounds and tannins from the skins and other grape materials during red wine making.

An optional procedure for white wines involves low temperatures (between 4-10 ^{o}C) that helps the wines to be characterised by more complex flavours. In white wines this procedure called "skin contact"

For red wines, the maceration procedure can end when alcoholic fermentation stops and makes the wines rich in colour and tannins (Robinson, 2006).

The inoculation of the must during winemaking is another optional procedure. There has been much discussion over the years concerning the relative merits of spontaneous versus induced fermentation. That various strains of S. cerevisiae supply distinctive sensory attributes are indisputable (Cavazza *et al*, 1989; Grando *et al*, 1993). Nevertheless, strain choice can equally affect the varietal character of aromatically distinctive cultivars, by influencing the liberation of bound grape flavourants. This may be particularly significant with non-*Saccharomyces* yeasts. These appear to have

greater activity in breaking glycosidic bonds (Mendes-Ferreira et al., 2001). Nevertheless, using established strains provides the winemaker with the greatest confidence that fermentation will be rapid and possess relatively predictable flavour and quality characteristics. Desired *S. cerevisiae* characteristics for winemaking include: osmotolerance, relative insensitivity to high acidity, and acceptance of low oxygen concentrations. When this yeast becomes adapted to the environment of fermenting grape must, it should be able to exclude other competitor microbes. Fermentation represents energy releasing metabolism in which both the substrate and the product are organic compounds. The difference with respiration is the requirement for molecular oxygen. Especially in alcoholic fermentation the substrate is D-glucose and the primary products are ethanol and carbon dioxide.

In contrast, spontaneous fermentations may accentuate yearly variations in character. It can be part of the uniqueness (mystique) associated with *terroir*. These elements are often desirable (or essential) in marketing premium wines. However, it also carries the risk of conferring off -odours or other undesirable traits. Occasionally, but not consistently, spontaneous fermentations generate higher concentrations of volatile acidity than induced fermentations. Spontaneous fermentations also tend to possess noticeable lag periods (most likely due to the low inoculum of *S. cerevisiae*) and, thus, are more susceptible to disruption by killer factors. Those who favour spontaneous fermentation believe that the indigenous grape flora supplies a desired subtle or regional character (Mateo *et al.*, 1991),

supposedly missing with induced fermentations. Large-scale wineries, where brand-name consistency is essential, cannot take risks with spontaneous fermentation. Nonetheless, even induced fermentations are not pure-culture fermentations. The juice or must always contain a sizable population of epiphytic yeast and bacteria from the grapes, unless pasteurized or treated to thermovinification.

An alternative to either spontaneous or standard induced fermentation is inoculation with a mixture of local and commercial yeast strains (Moreno *et al.*, 1991). The combination appears to diminish individual differences, producing a more uniform and distinctive character. This may also involve the joint inoculation with species, such as *Candida stellata* (Soden *et al.*, 2000) or *Debaryomyces vanriji* (Garcia *et al.*, 2002). Another choice for winemakers searching to add a distinctive aspect to their wine is the use of cryotolerant yeasts, primarily *S. uvarum*. *S. uvarum* is characterized not only by its ability to ferment at temperatures down to 6 °C, but also by its potential to synthesize desirable sensory characteristics. For example, cyrotolerant yeasts generally produce higher concentrations of glycerol, succinic acid, 2-phenethyl alcohol, and isoamyl and isobutyl alcohols; synthesize malic acid; and produce less acetic acid than many mesophilic *S. cerevisiae* (Castellari *et al.*, 1994; Massoutier *et al.*, 1998).The impact that yeast has on wine should not be underestimated in terms of modulation of aesthetic and organoleptic properties such as appearance, bouquet, flavour, taste, and mouth-feel (Lambrechts and Pretorius, 2000). Research strives to understand in detail how the

difference in fermentation behaviour and wine flavour composition between yeast strains influences winemaking and wine quality.

Research has focused on genetic approaches of industrial yeast characterization (Attfield and Bell, 2003). With the development of high-throughput DNA sequencing technologies 46 of most the industrial yeast strains genomes are now available (Piskur and Langkjaer, 2004). Molecular genetic research has also included studies of gene expression in wine yeast during fermentation (Backhus, 2001, Marks, 2003, Rossignol, *et al.* 2003), when subjected to stresses like high sugar concentration (Erasmus, 2003) or ethanol toxicity (Alexandre, *et al.*, 2001) and when yeasts are dehydrated-rehydrated (Rossignol, *et al.*, 2006).

Regarding yeast growth during wine fermentation, this usually occurs in batch fermentation mode. That means nutrients are available at the beginning of the fermentation and decline gradually when the alcohol concentration increases towards the end. Batch fermentation in most cases may be described by four phases; lag, log, stationary, and decline (see Fig 2.).

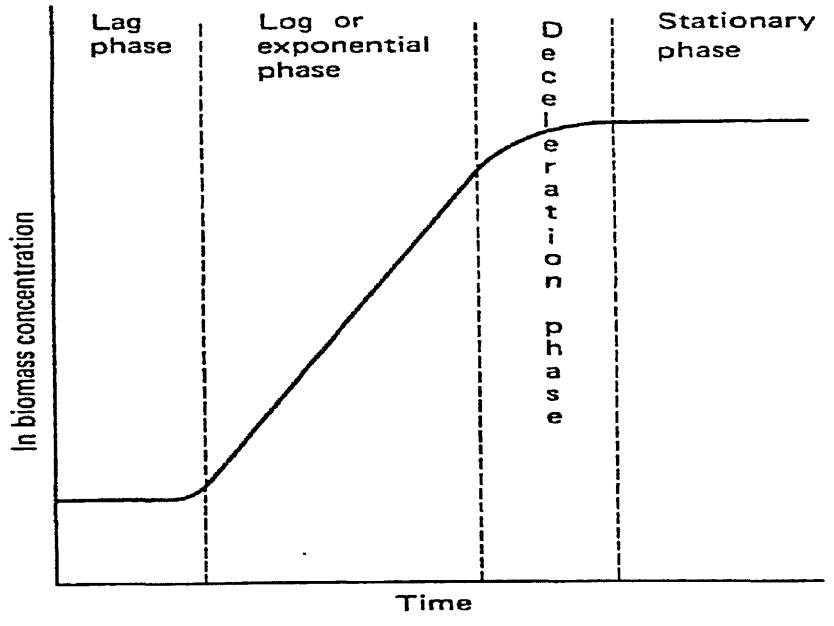


Figure 2 Growth of a typical microbial culture in batch conditions

Immediately after inoculation there is a period of time during which it appears that no growth takes place and that happens because yeasts cells need to adapt to the new environmental conditions This period of time is referred as lag phase. Following the adaptation to the new environmental conditions, the population of viable cells will increase (acceleration phase) according to the equation: $D\chi/dt = \mu \chi$

The value of specific growth rate (μ) varying between 0 (lag phase) and μmax (exponential phase).

During fermentation and after the log phase the nutrient concentration falls and the alcohol concentration increases. At the same time the toxicity of the bioproducts increases and the number of viable and metabolically active cells approaches the number of dead cells. The

culture now enters the stationary phase. This involves considerable transcriptional modification (Rossignol *et al.*, 2003). In industrial situations such as wine fermentations, the lag phase is of variable duration, the exponential growth phase is very short, the stationary phase may be short and commence long before the substrates become exhausted and the decline phase is long with the total number of viable cells remaining at high levels for several months (Ribereau-Gayon, 1985). When yeast is first inoculated into fresh grape must, physiological changes to the yeast may appear to be minimal. This is because conditions such as low temperature and wine must "sulphiting" (addition of sulphur dioxide as an antioxidant and antimicrobial agent) during grape crushing protects yeast from oxygen (Kraus *et al.*, 1981). However, the addition of sulphur dioxide for antimicrobial protection has been shown to have a negative affect on yeast growth (Ough, 1966a). In addition, nitrogen deficiency (eg. below 150 mg/l) and low pH (<3.3 for white wines and < 3.4 for red wines) can extend the lag phase. High sugar concentrations (up to 250 g/L) at the beginning of fermentation also inhibits yeast growth and fermentation ability (Ough, 1966a, b).

The next step in wine fermentations is the exponential phase when the yeast cells grow and reproduce at a maximal rate permitted by the prevailing conditions. In this stage the presence or the absence of oxygen does not affect the yeast growth rate (Schulze *et al.*, 1996). RNA and protein content in the cytoplasm rise and glycerol and trehalose accumulate and this has significance for yeast stress

physiology, as discussed below. As yeasts cells enter the stationary phase there is a change in the yeasts' enzyme complement; several stress proteins (heat shock proteins, Hsp) are produced and intracellular levels of glycerol and trehalose are elevated to act as stress protectant molecules (Riou *et al.*, 1997). Trehalose stabilizes membrane fluidity (Iwahashi *et al.*, 1995) and limits protein denaturation (Hottiger et al., 1994), whilst glycerol plays an important role for the maintenance of intracellular redox balance. It has been reported that glycerol synthesis, at least during the stationary phase, is associated with redox balance by eliminating excess reducing power (Roustan and Sabrayrolles, 2002). Heat shock proteins produced by several kinds of organisms produce the same result as above (Parsel *et al*,. 1994).

During the decline phase, yeast membrane dysfunction increases under the combined effect of ethanol and mid chain fatty acid toxicity (Hallsworth 1998; Viegas *et al.*, 1998).

But what happens (in terms of yeast stress) during inoculation of grape must in industrial conditions?

Yeasts cells are usually added to wine must to achieve an initial population cell density of about 10^5-10^6 cells/ml. When dried yeast preparations are used (as is common practice), this is equivalent to about 0.1-0.2 g yeast/L of must. Industrial dried yeast often contains about 20-30 x 10^9 cells/g. Before adding the dried yeast, the inoculum is prepared by placing the yeast in warm water at temperature of 38-40°C which is optimal for cell rehydration (Kraus *et al.*, 1981). Continuously cooling to 25°C is followed for a short period of

adaptation. During this time cellular metabolism and membrane permeability readjust to normal. This period of time is the most important period for cells before they are exposed to the high osmotic environment of the grape must. The re-adaptation involves transcriptional activation of about 2000 different genes (Rossignol *et al.*, 2006).

1.2 Wine yeast stress phenomena

The following section describes in more detail the influence of major stress factors encountered by wine yeasts during fermentation. This are depicted in Figure 3 and include:

- Sugars
- Sulphur dioxide
- Oxygen
- Carbon dioxide
- pH
- Temperature
- Ethanol

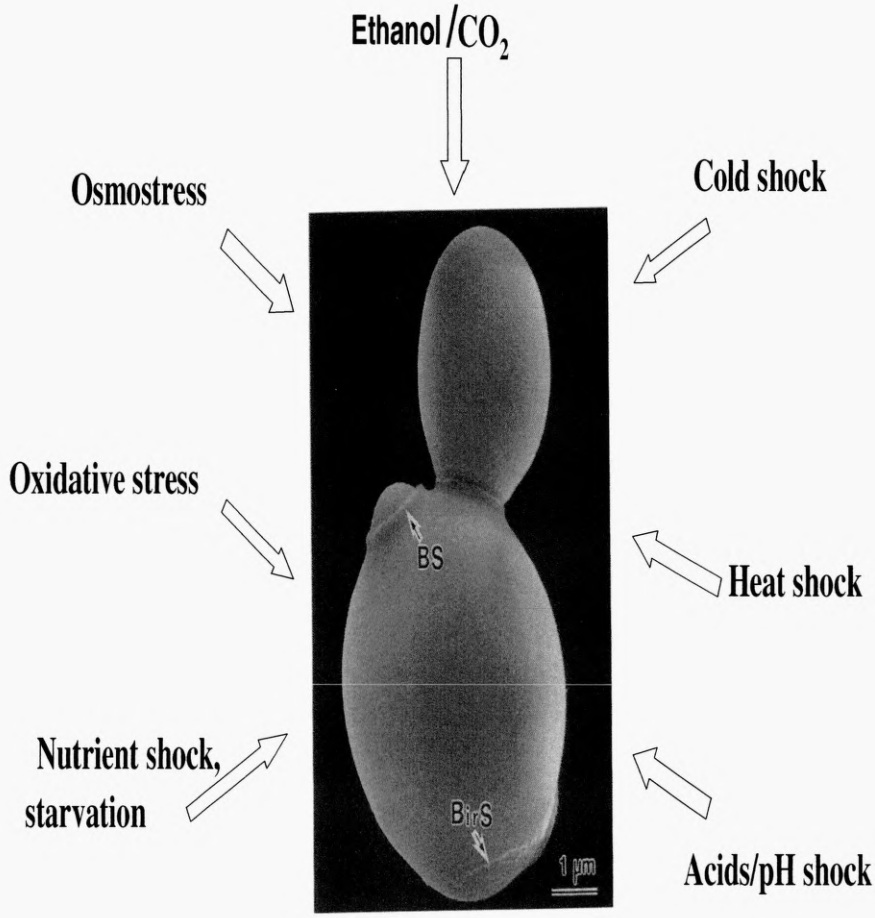


Figure 3 Stress factors that encountered by wine yeasts during fermentation (adapted from Walker,G.M., Yeast Physiology and Biotechnology 1998)

1.2.1 Influence of sugar on wine yeast fermentation

The major carbon and energy sources in grape must are glucose and fructose. Other nutrients such as amino acids may also be utilized in this manner but they are present in small amounts and only glucose, fructose and sucrose can be fermented easily by yeasts. Glucose in the early stages of fermentation is translocated across the plasma membrane by several transport mechanisms (Kruckeberg, 1996). Some of these are low-affinity systems that typically work at high substrate concentrations. Glucose concentration not only differentially affects the activation of sugar transport systems but also regulates expression of enzymes in the tricarboxylic acid (TCA) cycle and glyoxylate pathways. At the same time continued expression of selected chromosomal and mitochondrial genes results in some TCA enzymes being found in the cytoplasm, even at high sugar concentrations. They are required for biosynthetic reactions essential for growth. In mature grapes, the sugar concentration easily reaches 20 to 25% w/v. At this concentration the osmotic influence of sugar can delay the onset of fermentation. The partial plasmolysis of yeast cells may be one of the causes of a lag period prior to active fermentation (Nishino *et al.*, 1985). Additionally, cell viability may be reduced, cell division retarded, and sensitivity to alcohol toxicity enhanced. At sugar concentrations higher than 25% w/v, the likelihood of fermentation terminating prematurely increases considerably. The adaptation and the tolerance of the wine yeasts to high sugar concentrations is related to increased synthesis of glycerol and the permeability of the cell membrane (Brewster *et al.*, 1993).

These responses to increased osmolarity permit glycerol to equilibrate the osmotic potential of the cytoplasm to that of the surrounding grape juice. High sugar concentration increases the productivity of acetic acid (Schanderl, 1959) and acetate esters. Additionally, ethanol production, for concentrations above 30%w/v of sugars, starts to decline (Henschke and Dixon, 1990). Section 1.3.1 discuses in more detail yeast response to osmostress caused by high levels of sugar and other factors such as high salt.

1.2.2 The roles of sulphur dioxide in winemaking

Sulphur dioxide may be added to grape must at a level between 50-100 mg/L depending on the health of the grapes and the maceration temperature. Recent studies have thrown into question the benefits of the use of SO_2 especially for healthy grapes macerated at cool temperatures. The addition of sulphur dioxide during wine making not only favours the growth of strains resistant to sulfur dioxide but also appears to select strains that produce greater amounts of sulphur dioxide. SO_2 is antioxidant and disinfectant, and is used at many stages in winemaking. To prevent fermentation starting prematurely, it may be added to inhibit the growth and metabolism of wild yeasts and bacteria. These organisms require oxygen for growth, and are naturally found on grape skins. In general, the uses of sulphur dioxide focus on the following aspects:

1. Antiseptic:

It inhibits the development of microorganisms. It has a greater activity

on bacteria than on yeasts. At low concentrations, the inhibition is transitory. High concentrations destroy a percentage of the microbial population. The effectiveness of a given concentration is increased by lowering the initial population, by filtration for example. During storage, SO_2 hinders the development of all types of microorganisms (yeasts, lactic acid bacteria, and, to a lesser extent, acetic acid bacteria), prevents yeast haze formation, and secondary fermentation of sweet white wines. It also inhibits *Brettanomyces* (wild yeast causing wine flavor defects) contamination and the subsequent formation of ethyl-phenols, the development of other wild yeasts strains that might still exist in wine (flora) and various types of bacteria spoilage .

2. Antioxidant:

The interaction of SO_2 with oxygen has been studied for over one hundred years, mainly because of its importance in the desulphurization of flue gases and production of acid rain in the atmosphere, where pH is ~4; not too distant from that of wine (Brandt and van Eldik 1995). In aqueous systems SO_2 combines with water to produce sulphurous acid, which dissociates as shown in reaction 1, such that in wine the bisulphite form (HSO_3^-) predominates. The reaction with oxygen may be represented by reaction 2, where two moles of bisulphite react with one mole of oxygen to produce two moles of sulphate. However, drawn in this manner, it could imply that bisulphite reacts directly with molecular oxygen, which is highly misleading. The actual mechanism by which it is converted to sulphate is quite complex, which has important implications regarding the chemical transformations it can induce in

wine. There is a fundamental reason why oxygen cannot interact directly in one step with SO_2 or bisulphite and sulphite ions. The outer electrons of the oxygen molecule that are available for forming covalent bonds are in separate molecular orbital with their spins aligned. In normal conditions, therefore, molecular oxygen is in a triplet ground state; in effect a di-radical. Such a molecule cannot interact directly with species such as polyphenols and SO_2, which possess paired electrons with antiparallel spins (singlet state). If this reaction did occur, it would result in new molecular orbital containing electrons with parallel spins, which would violate Pauli's exclusion principle. The reaction could only proceed with spin inversion to the singlet state, which would require a substantial energy input. Without such input, reactions with oxygen proceed in one-electron steps with the formation of free radicals (Danilewicz, 2003).

$$H_2O + SO_2 \underset{pK_a = 1.86}{\rightleftharpoons} HSO_3^- + H^+ \underset{pK_a = 7.2}{\rightleftharpoons} SO_3^{2-} + 2\,H^+ \qquad (1)$$

$$2\,HSO_3^- + O_2 \longrightarrow 2\,H^+ + 2\,SO_4^{2-} \qquad (2)$$

This reaction is slow. It protects wines from chemical oxidations, but it has no effect on enzymatic oxidations, which are very quick. SO_2 protects wine from an excessively intense oxidation of its phenolic compounds and certain aroma chemicals. It prevents madeirization

which is the reaction of phenolic compounds with oxygen and transforms the color of white wines from light yellow to dark yellow and in red wines from dark red to dark slate color. It also contributes to the establishment of a sufficiently low oxidation– reduction potential, favoring wine aroma and taste development during storage and aging.

3. Anti-oxidase:

It instantaneously inhibits the functioning of oxidation enzymes (tyrosinase, laccase) and can ensure their destruction over time. Before fermentation, SO_2 protects musts from oxidation by this mechanism. It also helps to avoid oxidative reactions in white and red wines made from rotten grapes.

4. Flavor protectant:

Binding ethanol and other similar products, SO_2 protects wine aromas and makes the flat character disappear.

5. Miscellaneous roles:

SO_2 does not appear to affect the rate of alcoholic fermentation at the concentration typically used (50ppm), but can slow the onset of fermentation. The presence of 15-20ppm can reduce yeast viability from 10^6 to10^4 cells/ml or less (Lehmann, 1987). Yeast resistance to sulphur dioxide is correlated with several factors such as the *SSU1R* gene that controls sulphite efflux. (Hauser *et al.*, 2001). Additionally, SO_2 can affect yeast metabolism by binding with several carbonyl compounds like acetaldehyde, pyruvic acid and α-ketoglutaric acid.

1.2.3 The roles of oxygen in wine fermentation

Even if fermentation itself is anaerobic process and requires no oxygen, trace amounts of oxygen favour fermentation by permitting the biosynthesis of sterols and long chain unsaturated fatty acids. The proper functioning of the yeast plasma membrane requires sterols as well as C_{16} and C_{18} fatty acids. Additionally, anaerobic conditions favour the accumulation of toxic C_8 and C_{10} fatty acids (Alexander and Charpentier, 1995). During winemaking wine must becomes oxygenated following pre-fermentation treatments such as de-stemming, crushing, skin contact, cryo-extraction and inoculation of the must. Oxygen promotes yeast growth and fermentation and diminishes the reductive influence (Fornairon-Bonneford *et al*, 2003).

1.2.4 Carbon dioxide production during fermentation

It is well known that during alcoholic fermentation large quantities of carbon dioxide are produced. This amount approaches 260 mg/g of fermented glucose. In most cases, sugar concentrations of must are about 200-230 g/L and the amount of carbon dioxide produced during an alcoholic fermentation with this concentration is nearly 50 times the volume of the juice fermented. During the production of carbon dioxide various volatile compounds are carried off and ethanol loss is estimated at about 1-2% of that produced, although this varies with sugar and fermentation temperature (William and Boulton, 1983). For example,

higher alcohols, monoterpenes, ethyl and acetate esters may be lost and this is strongly dependent on fermentation temperature (Miller *et al.*, 1987).

In some recent wine making practices fermentation is conducted at low temperatures, and especially in the range between 8°C and 14 °C. Under these conditions, the escape of carbon dioxide is enhanced and the pressure in fermentation tanks rise. At pressures around 30kPa, yeast growth rate ceases and together with low pH and high alcohol content the sensitivity of the yeast increases (Kunkee and Ough, 1996). In addition, carbon dioxide may affect the balance between carboxylation and decarboxylation. At the same time water viscosity may be affected (Bett and Cappi, 1965). Critical CO_2-induced changes in membrane composition may occur and can disrupt yeast membrane permeability. The presence of heat shock proteins and trehalose can limit protein denaturation and stabilize membrane fluidity during CO_2 exposure (Iwahashi *et al.*, 1995).

1.2.5 pH and wine fermentation

pH has normally little influence on fermentation rate or on synthesis and release of aromatic compounds during wine making. However, low pH may assist the uptake of some amino acids (Cartwright *et al.*, 1989). The most important effects of pH on fermentation are indirect such as the antimicrobial action of sulphur dioxide. Additionally, pH affects the production of some fermentation by-products such as the hydrolysis of ethyl and acetate esters. Several authors found a

significant improvement of secreted products yield at pH around 3, very close to wine pH, which was explained by host cell protease inhibition (Jahic *et al.*, 2003a). Also some yeast species (*Zygosaccharomyces*, *Pichia*) seem more acid tolerant than the others (Sousa *et al.*, 1996; Praphailong and Fleet, 1997) In *S. cerevisiae* five genes have already identified that they have an important role to growth adaptation under weak organic acids . The PDR12 gene encoding an ATP-depending membrane transporter is induced and repressed at low pH values(Causton *et al.*, 2001). *ZMS*1 and *TRK*2 (encoding a zing-finger family transcription factor and a potassium transporter, are activated by low and repressed under high pH value. Actually *TRK*2 is one of the genes which are represented in Table1 as one of the genes which is induced under NaCl stress. *CIT*2 (peroxisomal citrate synthase) and *PHO*89 (sodium phosphate symporter) are repressed at low and activated at high pH (Ko *et al.*, 1990). An important effect of weak acid stress is on the plasma membrane H^+-ATPase, an ATP driven proton pump(Pma1p) (Piper *et al.*,2001).

It has been reported that low pH induces changes in the organization of the cell wall of *S.cerevisiae* dependent on the HOG (high osmolarity glycerol) pathway. Upon acidic stress several cell walls proteins (*CWP*1, *HOR*7, *SP*11) and a secondary glycoprotein (*YGP*1) were induced (Kapteyn *et al.*, 2001). At low pH, like wines, weak acids enter the cell by passive diffusion. The higher intracellular pH dissociates weak acids, generating protons and acid anions that accumulate

intracellularly. Hence, weak acids show relatively strong antimicrobial effects at low pH, that are generally attributed to the release of protons and subsequent cytoplasmatic acidification, which inhibit essential metabolic functions (Krebs *et al.*, 1983). In *S.cerevisiae* the stress response is triggered by weak acids mediated via the transcription factor War1p, directly up regulating *PDR*12 expression (Kren *et al.*, 2003). It has been reported that *S.cerevisiae* accumulates trehalose in low pH in the presence of weak organic acids just like under osmotic stress conditions (Cheng *et al.*, 1999).

1.2.6 Temperature stresses in wine fermentation

Temperature has direct and indirect influences on yeast metabolism but it is also one of the fermentation parameters over which winemakers have the greatest control. Both low and high temperature can affect yeast cell physiology. Relative tolerance to high temperatures appears to depend on production of Hsp 104 heat shock protein (Parsel *et al.*, 1994) and this limits the aggregation of cellular proteins in the presence of ethanol and certain fatty acids. Low temperatures tend to diminish the toxic effects of ethanol and this is a consequence of higher proportions of unsaturated fatty acids residues in the plasma membrane.

The growth rate of yeast cells is influenced particularly during exponential phase and, more specifically, cell division seems to change depending on fermentation temperature (Charoenchai *et al.*, 1998). Cells exposed to temperatures above 20°C experience a rapid decline in

viability, especially at the end of the fermentation. In cooler environments, cell growth is retarded but viability enhanced. Cool temperatures also prolong the lag phase. Additionally, rehydration temperatures of dry yeast cells is critical (Llaurado *et al.*, 2005). Cool temperatures dramatically slow the fermentation rate and in some cases can lead to its premature termination. Excessively high temperatures may also induce stuck fermentation by disrupting enzyme and membrane function.

Modern winemaking favours cool temperature fermentations due to the fact that they can produce more fresh and fruity wines. Esters such as isoamyl, isobutyl and hexyl acetates are synthesized and retained to a greater degree at cool temperatures. A greater production of higher alcohols may also be observed under low temperature fermentations. In addition, the release of yeast colloids is reduced, thereby facilitating wine clarification. For red wines, fermentation can be conducted at higher temperatures; for example, between 25-30 °C which is typical temperature range increasing yeast growth rate and alcohol productivity. In addition, warmer temperatures are generally preferred due to positive effects on extraction of phenols and especially the extraction of anthocyanins and tannins which are the main chemical compounds which affects to wine color and taste.

1.2.7 Influence of ethanol toxicity in yeast fermentation

The major end product of fermentation of sugars is ethanol. A lot of research has been made regarding the mechanism of ethanol toxicity and ethanol tolerance of wine yeasts cells. Several factors appear to be associated with ethanol tolerance. These include: activation of glycerol and trehalose synthesis (Hallsworth, 1998), accumulation of Hsp104 and Hsp12 (Sales *et al.*, 2000) and modification of the plasma membrane. Regarding the latter, ethanol influences: activation of plasma membrane ATPase, substitution of ergosterol for lanosterol, the proportion of phosphatidyl inositol versus phosphatidyl chlorine (Arneborg *et al.*, 1995) and augmenting the incorporation of palmitic acid. These membrane changes decrease permeability (Mizoguchi and Hara, 1998) and minimize the loss of nutrients and cofactors from the cell, especially magnesium and zinc (Walker & Van Dijck, 2005; Walker *et al.*, 2006).

Vacuolar membrane function is also crucial for the retention of toxic substances stored in vacuoles (Kitamoto, 1989). In general, alcohol inhibits fermentation and it begins disrupting yeast metabolism at low concentrations (Dittrich, 1977), although most industrial strains of *S.cerevisiae* can ferment up to 13-15% v/v ethanol. It is generally believed that ethanol toxicity disrupts the semi fluid nature of the cell membrane by the yeast lowering water activity (Hallsworth, 1998). These changes to the yeast plasma membrane lead to a destruction of the ability of cells to control cytoplasmic function,

uptake nutrients and maintain the electrochemical gradient across the membrane (Cartwright *et al.*, 1989).

1.3 Yeast cells and NaCl-induced stress

This thesis is concerned with investigating the influence of NaCl on wine yeast physiology and fermentation performance. The following section therefore provides some background information on yeast osmostress and specific information on NaCl-induced stress.

1.3.1 Yeast osmostress- general responses

Yeast cells have been employed as organisms for studying the mechanisms underlying osmotic stress in general, and saline stress in particular. This is due to the fact that yeast possess similar ion transport systems to higher plants and fungi, and have similar detoxification mechanisms and signal transduction pathways.

Yeast stress responses can be distinguished at different stages such as:

1. Immediate cellular changes that occur as a direct consequence of the physico-mechanical forces operating under those conditions;
2. Primary defined processes and
3. Changes in cell homeostasis as a result of the new osmotic environment.

When yeast cells are exposed to osmotic stress, a number of

physiological changes take place. These include: efflux of intracellular H_2O, rapid reduction in total cell volume, including the vacuole (Blomberg and Adler, 1992), a transient increase in glycolytic intermediates (Singh and Norton, 1991), accumulation of glycerol in the cytosol (Brown 1978), and triggering of the HOG (Hyper Osmotic Glycerol) signaling pathway (Albertyn *et al.*, 1994). Microorganisms such as *S. cerevisiae* develop systems to counteract the deleterious effects of osmotic stress due to salt (NaCl) stress. More specifically, salt stress creates two different phenomena: ion toxicity and osmotic stress. Defense responses to salt stress are based on osmotic adjustments by osmolyte synthesis and cation transport systems for sodium exclusion. Polyols and especially glycerol are the major osmolytes produced by yeasts. Although it is well established that during saline stress cells must keep in balance the ratio between Na^+ and K^+ which depends on the plasma membrane Na^+ / K^+ ATPase , a P-type ion pump that drives Na^+ ions out of the cell and K^+ ions into the cell. Several pathways appear to mediate cellular Na^+ homeostasis in yeast cells (Posas *et al.*, 2000). One route of Na^+ entry is thought to be the K^+ transporters *TRK*1 and *TRK*2. But *TRK*1 is the gene which limits the entry of the anions Na^+ and K^+ (Lans *et al.*, 1997; Serrano *et al.*, 1997). It is important to consider that the quantitative effect on cell turgor or volume during growth in a medium by osmotic dehydration (caused by stress conditions such as sodium chloride) will differ between different yeast species (Hamilton *et.al.*, 2002). In *S. cerevisiae* cells, glycerol

is produced as a "compatible solute" via metabolic pathways summarized in Figure 4.

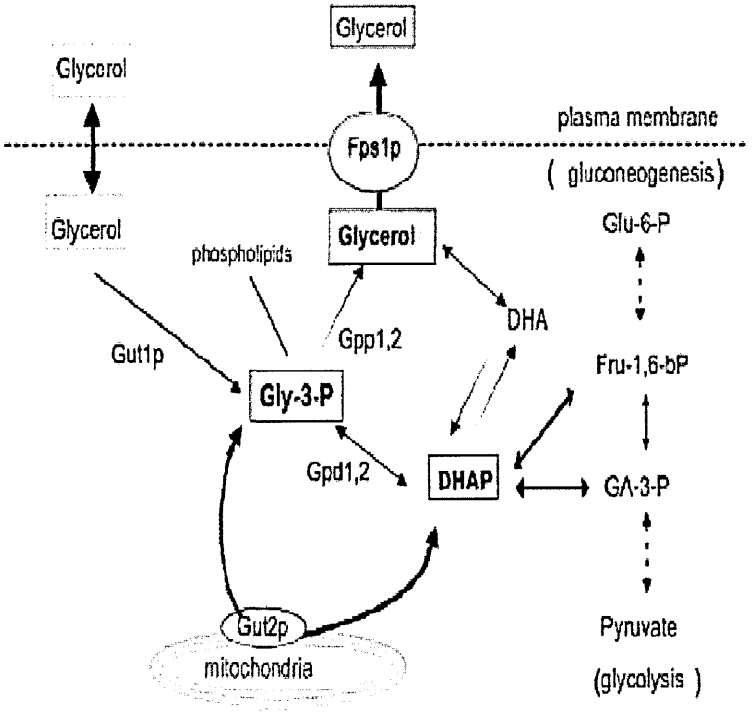


Figure 4. The input and output mechanism of glycerol in yeast cells during osmotic stress (adapted from Blomberg and Adler, 2000)

Another compound produced during stress conditions is trehalose that, together with glycogen may represent 25% w/w of the yeast dry cell mass depending on the environmental conditions. The disaccharide trehalose accumulates during salt adaptation (Blomberg and Adler 2000; Parrou *et al.*, 1997) and has also been shown to protect cells against high temperature by stabilizing proteins (Singer and Linquist, 1998) and against cellular desiccation by helping to both stabilize proteins (Allison *et al.*, 1999) and to maintain membrane integrity (Growe *et al.*, 1984). Exposing yeast cells to a

hyper osmotic environment or medium leads to a rapid initial cellular efflux of water into the medium, which, in other words, is cell dehydration. Dehydration is a rapid process and takes place in about a minute for most cells. The efflux of water is mediated solely through the lipid bilayer. In addition, intracellular water is recruited from the vacuole into the cytoplasm thus partially compensating for the sudden increase in macromolecular concentration. The yeast cytoskeleton also collapses leading to depolarization of actin patches. This cell dehydration and loss of the cell water leads to a growth arrest. Under these conditions cellular reprogramming is the main defence mechanism. The cellular reprogramming is synonymous with cell adaptation. During this reprogramming most cells accumulate compatible solutes to balance the intracellular osmotic pressure with the external environment. The compatibles solutes can be: glycerol, trehalose, amino acids, and fatty acids in the cell membrane.

The role of glycerol and trehalose are described in more detail below.

1.3.2 Glycerol and yeast osmostress

Glycerol is a polyhydroxy compound which may accumulate at very high concentrations inside yeast cells without toxic or inhibitory effects (Brown 1978). Accumulation of glycerol correlates with decreased water potential of the medium. Under osmostress

conditions, glycerol production is proportional to the osmotic stress (Nobre & da Costa, 1985; van Zyl & Prior, 1990; Olz *et al.*, 1993) and can represent up to 25% of the dry cell mass (Lillie & Pringle, 1980). Glycerol is the most prominent compatible solute and a growing amount of evidence indicates that the intracellular level of glycerol is adjusted to external water activities.

The most commonly used osmolyte in experiments to cause hyper osmotic stress is sodium chloride. It has been established that the intracellular concentration of glycerol increases in parallel to the external concentration of sodium chloride. In general, an increase in the intracellular concentration of glycerol can be the result of increased synthesis, increased cytoplasmic retention, or decreased dissimilation or uptake of glycerol from the medium. Glycerol is produced during glycolysis by reduction of dihydroxyacetone phosphate to glycerol 3-phosphate by glycerol 3-phosphate dehydrogenase (GPD) (Reed et al,. 1987; Bellinger and Lonher, 1987; Meikle *et al.*, 1988). Under osmotic stress, increased levels of glycerol take place due to the increase of the activity of (cytosolic) ctGPD. This glycerol formation requires an equimolar amount of cytoplasmatic NADH. When cells are osmotically stressed, this requirement seems to be partially met by decreased reduction of acetaldehyde to ethanol on the one hand and an increased oxidation to acetate in the other. The observed decrease in the synthesis of alcohol dehydrogenase as well as the increase of the aldehyde

dehydrogenase could account for this alteration in flux. The key enzyme (cytosolic) ctGPD is strongly induced via specific signal transduction pathways such as HOG (high osmolarity glycerol) and HOGMAPK (high osmolarity glycerol mitogen activated protein kinase) (Blomberg and Adler, 1989; Brewster *et al.*, 1993; Elion 2000; Hohmann 2002; Van Uden 1985; Attfield 1987).

1.3.3 Trehalose and yeast osmostress

Trehalose is an important non-reducing disaccharide and, in addition to its function as a storage carbohydrate, it has also been recognized as one of the most important stress protectants in yeast cells It is formed by an α 1,1 linkage of two D-glucose molecules (see Fig 5). The molecular formula and weight are $C_{12}H_{22}O_{11}$ and 342.31, respectively. Trehalose has a high degree of optical rotation and melts at 97°C. Additionally heat drives off the water of crystallization until the material resolidifies at 130°C and then the anhydrous trehalose melts at 203°C. The α,α form is the isomer which referred to as trehalose (α,α- trehalose, α-D-glucopyranosyl α-D-glucopyranoside, or mushroom sugar, or mycose) is widespread through the plant and animal kingdoms. It is the one of the most effective protectant molecules for cell membranes under osmotic stress conditions and helps to prevent any damage to the lipid bilayer. That kind of protection occurs because trehalose decreases the thermotropic phase transition of membrane lipids in the dry state to maintain the permeability of membrane lipid bilayer. This is the second cell response under osmotic stress. Production and

accumulation of trehalose also occurs during thermal stress, substrate starvation, oxidative stress, chemical stress and cold stress.

Basically, the protective effect of trehalose has been expressed via two different hypotheses:

1. According to the first hypothesis trehalose replaces water molecules that are hydrogen bonded at the surface of biological macromolecules and they are essential. That occurs because hydrogen binds trehalose with many hydroxyl groups facilitating their bonding stability under conditions such as freezing, osmotic stress, heat and desiccation compared to hydrogen bonding with water molecules.

2. The second hypothesis is based on the tendency of trehalose to undergo glass rather crystal formation during desiccation conditions. The glass capsule around macromolecules would freeze their native shape and would prevent any distortion of their structure during dehydration.

Previous work has shown that trehalose uptake is linear with time and accumulates in the cytoplasm. Recent research has shown that a subunit of trehalose-6-phosphate synthase/phosphatase complex is involved in glucose efflux during glycolysis.

The trehalose content of commercially available yeast is widely believed to be a critical element for their stress resistance and has received a great deal of attention particularly regarding the production of "instant dry yeast" and the preservation of yeast viability in frozen dough. The presence of sodium chloride in the culture medium elevates trehalose accumulation (Carvalheiro *et al.*,

1999). It has been reported that under hyper saline conditions, yeast cells increased their trehalose content to withstand higher ethanol conditions. It is believed that ethanol interacts with the membranes by inserting into hydrophobic regions increasing polarity, thereby weakening the hydrophobic barrier to the free exchange of polar molecules and affecting the disposition of membrane components (Attfield, 1987). Yeast cells with high intracellular concentrations of trehalose are tolerant to adverse environmental conditions such as saline conditions (Sukesh, 1997). A high content of trehalose also protects cells from autolysis and increases leavening capacity in dough (Attfield, 1997; Quain, 1998; Randez *et al.*, 1999). Trehalose fulfils two unique properties that make this molecule a stress protectant.

Firstly, is the capacity of trehalose to protect membranes from desiccation. This action is known as "the water-replacement hypothesis" (Growe *et al.*, 1999). A second important and complementary function of this disaccharide is its ability to exclude water from the protein surface and hence to protect proteins from denaturation in dehydrated cells. A high level of trehalose can protect native proteins from denaturation and also suppress the aggregation of denatured proteins, which prevent their subsequent refolding by molecular chaperones (Parrou and Fracois, 2001). The increased production of trehalose is reflected in the biosynthetic pathway from glucose-6-phosphate to trehalose (see Fig 5). During salt stress a dramatic increase of trehalose-6-

phosphate is revealed. This compound has an inhibitory action on two of the three isoforms of the first enzymatic step in glycolysis, the hexokinases (Carvalheiro *et al.*, 1999). Trehalose biosynthesis has been reported to be different in various organisms. In yeasts, the pathway was elucidated more than 50 years ago (Cabib and Leloir.,1957). At the beginning of this pathway a glycosyl residue transfers from uridinedophospho-glucose to glucose 6-phosphate and provide trehalose-6- phosphate. The two enzymes that catalyze the trehalose biosynthesis are T6P synthase and T6P phosphatase. These two enzymes are part of a complex in which two other proteins Ts11 and Tps3 participate but without catalytic activity.

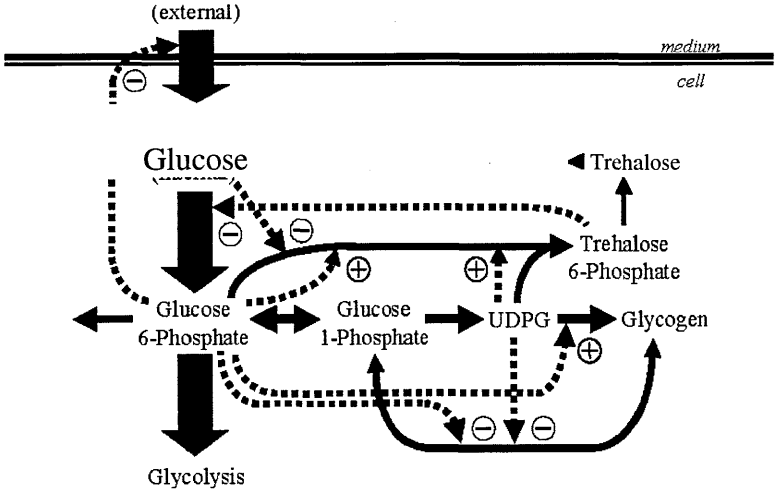


Figure 5 Trehalose biosynthesis by *S. cerevisiae* Solid arrows show flow of material, with thickness indicating the magnitude of flux. Dotted arrows represent inhibitory (-) and activating (+) signals (adapted from Voit, 2003)

1.4 HOG and MAPK pathways and yeast osmostress

The HOG pathway contains two transmembrane proteins, Sho1 and Sln1 that act on downstream proteins to ultimately regulate the MAPK pathway. These proteins have different functional domains and communicate directly to different downstream effectors proteins. These two HOG upstream branches under specific conditions appear to be redundant for growth in high osmolarity medium. On the other hand, activity of the Sln1 branch is required to induce the expression of several reporter genes in response to very high solute levels and this indicates that the Sln1 branch operates over a broader range of osmolarities than the Sho1 branch. Sln1 has two transmembrane regions and an intracellular histidine kinase domain that signals to two other proteins, Ypd1 and Ssk1, which together form a phosphorelay system. Phosphorelay systems comprise a histidine kinase protein that transfers a phosphate group to an intermediate protein, which then transfers the phosphate to a response regulator protein. In more detail, Sln1 under osmotic stress conditions leads to phosphorylation of the downstream target protein Ypd1, which continuously transfers a phosphate group to the response regulator protein Ssk1 that is the ultimate phosphor-acceptor in this phosphorelay system. At this point there is an interaction between Ssk1 and MAPKKKsSsk2 and Ssk22 and consequently the downstream components of the pathway remain inactive. After exposure to increased external osmolarity the histidine kinase activity of Sln1 is thought to be inhibited. Thus, Ypd1 and Ssk1 are

dephosphorylated and this enables binding of Ssk1 to MAPKKK Ssk2 triggering Ssk2 auto- phosphorylation and subsequent phosphorylation of Pbs2 and activation of Hog1.(Hohmann, S., Yeast Stress response, 2005) The Sho1 protein is composed of four transmembrane segments and a C-terminal SH3 domain through which interacts with downstream signaling elements in the HOG pathway. Sho1 functions have been found that can be completely bypassed by over expression of a membrane-targeted version of Pbs2 (Hohmann and Mager, 2003). Several findings from early 90's (Wienkem, 1990) suggest that Sho1 branch might not sense osmolarity directly but might instead provide a docking site for downstream proteins. In *S.cerevisiae,* this specific branch also uses components of the pheromone-response and filamentous growth MAPK pathway. This includes also Ste20 (a p21-activated kinase), Ste50 (a SAM domain-containing protein) and the MAPKKK Ste1 1. Functional Ste20 activates Ste11 by phosphorylation during pheromone signaling and also during increased osmolarity because the Ste11 phosphorylation sites are required for both responses. Ste50 might be a cofactor for Ste11 because these proteins form a complex through interaction of their SAM domains. Additionally, Pbs2 contains an N-terminal polyproline domain that can bind the SH3 domain of Sho1. A Pbs2 point mutant with a compromised SH3 binding site illustrates the importance of Pbs2-Sho1 interaction. Due to the fact that Pbs2 interacts with multiple proteins it has been proposed to act as a scaffold linking Sho1 to Ste11 activation and

thereby possibly limiting cross-talk to other Ste1 1-depented MAPK kinase pathways. Continuously in the nucleus Hog regulates the expression of numerous genes by controlling the activity of several transcription factors (activators and repressors). DNA studies indicate that Hog1 significantly regulates the expression of ~ 600 genes in response to increased osmolarity (Hohmann,2002)

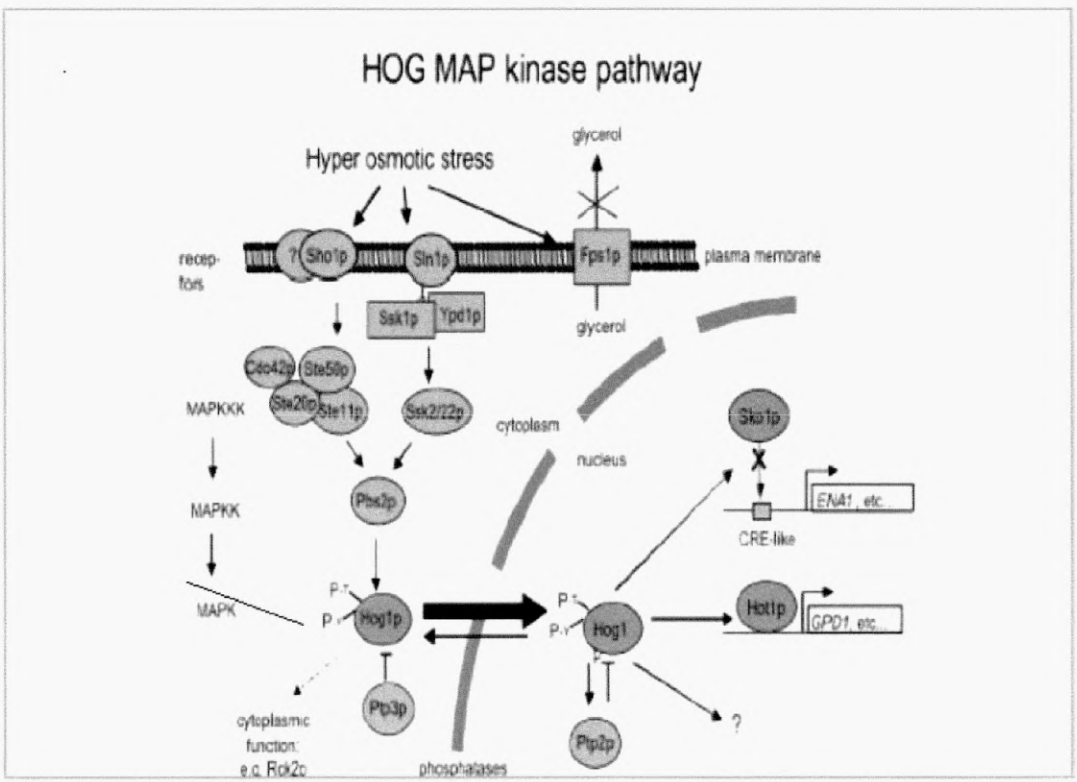


Fig.6 Protein expression and gene triggering during activation of HOG MAP kinase pathway under osmotic stress (adapted from Hohmann, 2002)

Sk1 binds DNA motifs upstream of several osmo-inducible genes and then recruits the general repressor complex Tup1-Ssn6 to repress

transcription. During elevated osmolarity, Hog1 phosphorylates Sko1, reducing the affinity of the protein for the Tup1. This allows transcription to proceed. Hot1 is a transcriptional activator that exhibits a two-hybrid interaction with Hog1 and also displays Hog1- and osmotic shock dependent phosphorylation. Mutation of HOT1 reduces but does not eliminate osmotic induction of the GPD1 and GPP2 genes, which control glycerol production. HOT1 and MSN1 mutations reduce GPD1 even further. It is interesting to mention that Hot1 is constitutively bound to the GPD1 promoter and that it recruits Hog1 to the DNA during osmotic stress. Msn1 is also localized at the GPD1 promoter but only during osmotic stress and in HOT1 strains (Mendizabal, *et al.* 1998) Msn2 and Msn4 are homologous zing-finger transcriptional activators that induce transcriptional activation during a variety of stresses including osmotic stress causes by NaCl. Maximal induction of Msn2 and Msn4-dependent genes during osmotic stress also requires Hog1 and additionally recruits both Hog1 and Hot1 to the CTT1 and HSP12 promoters further suggesting a functional interaction between the HOG pathway (Wiemkem, 1990). Msn2 and Msn4 transcription factors are the mediators of the general stress response. Under non-stressed conditions these two factors are localized in the cytoplasm but upon stress they rapidly translocate to the nucleus where they bind to the STRE-control elements in the promotion of a large number of stress responsive genes.

1.4.1 Cell activator proteins in yeast osmostress

Cell membranes have to allow passage of various polar molecules such as ions, sugars, amino acids, nucleotides and many cell metabolites that pass across synthetic bilayers only very slowly. For that kind of transportation special membrane proteins are responsible and are referred to as membrane transport proteins. There are two major categories of transport proteins: carrier proteins and channel proteins.

Carrier proteins (also called carriers, permeases, or transporters) bind the specific solute to be transported and undergo a series of conformational changes in order to transfer the bound solute across the membrane. Channel proteins need not bind to the solute and they form hydrophilic pores that extend across the lipid bilayer. When these pores are open they allow specific solutes, usually inorganic, to pass through them and thereby cross the membrane. All channel proteins and some carriers allow solutes to pass only passively ("downhill"). This process called passive transport or facilitated diffusion. Cells also require transport proteins that will actively pump certain solutes across the membrane against their electrochemical gradient ("uphill"). This process called active transport.

In the case of stress caused by sodium chloride the activator proteins for yeasts cells are the proteins belonging to the family of Yap (n) p activators proteins. Especially responsible for the response to osmotic stress is the fourth member of the family: Yap4p (Cin5p/Hal6p) and

Yap6p.

Several researches (Fernandes *et al.*, 1997; Wysocki *et al.*,2004; Rodrigues-Pousada *et al.*,2004) have shown that both YAP4 and YAP6 genes are triggered under osmotic, heat and oxidative stress conditions. Three other genes have been validated as dependent on Yap4p. Two of these are involved in glycerol biosynthesis: GCY1 encoding a putative glycerol dehydrogenase and GPP2 which encodes a NAD-depended glycerol3-phosphate phosphatase. Furthermore, DCS2 a gene homologous to the DCS 1-encoded decapping enzymes is the third gene which is activated by Yap4p (Pousada *et al.*, 2004).

1.4.2 Regulatory Genes and yeast osmostress (NaCl)

During osmotic stress induced by sodium chloride, the expression of glycerol-3-phosphate dehydrogenase encoded by the *GPD*1 gene is stimulated. This enzyme is always found to be induced by low water potential (Norberg and Blomberg, 1997). *ENA*1 is the gene of the sodium pumping ATPase and, during salt stress, is increased (Garciadeblas *et al.*, 1993; Norberg and Blomberg, 1997). Under the same conditions *ALD*2, *GTT*1, *HSP*104, *HSP*12 are expressed and members of the *HAL* gene family that appear to be involved as factors that will affect tolerance to increased osmolarities (Gaxiola *et al.*, 1992; Glaser *et al.*, 1993). The genes *GTT*1, *HSP* 104, *HSP*12 and *HSP*26 are heat shock genes but are strongly induced by osmoshock (Schuller *et al.*, 1994, Varela *et al.*, 1992). Other common aspects of heat and osmotic shock responses can be found with *MSN2* and *MSN4* gene

function. These genes encode zinc-finger proteins that specifically bind to stress response elements (STREs) (Martinez-Pastor *et al.*, 1996). STREs are present in a large number of genes induced by heat or osmotic stress. The *YAP*1 gene has been found to activate sequences containing STREs. In addition, the *ROX*1 gene has been found to be involved in heat and osmoshock response. *CYC*7 is another gene which is induced in heat and osmotically stressed cells and trehalose encoded by *CIF*1 which is a gene that encodes trehalose synthase complex (Iraxte et al., 1998). Yeast *HAL*1 and *HAL*3 genes apparently contribute to reduction of intracellular sodium levels by increasing *ENA*1 expression. *ENA/PMR*2 locus, a gene tandem array encoding isoforms of a putative P-ATPase (Iraxte *et al.*, 1998; Burg *et al., 1997*).

Table 1 Genes which are involved in osmotic stress response due to sodium chloride in *Saccharomyces cerevisiae* (from: www.ihop-net)

Symbol	Name	Synonyms
*ALD*2	Cytoplasmatic aldehyde dehydrogenase, involved in ethanol oxidation and beta-analine. biosynthesis; uses NAD^+ preferred coenzyme; expression in stress and glucose repressed	*ALD*5, *YM*8520, *YMR*170C
*ENA*1	P-type ATPase sodium pump, involved in Na^+ and Li^+ efflux to allow salt tolerance	*HOR*6, *PMR*2,*PMR*A, Sodium transport ATPase 1, YD6888, 02C, YDR040C
*ENA*2	P-type ATPase sodium pump, involved in Na + efflux to allow salt tolerance, likely not involved in Li^+ efflux	*PMR*2B Sodium transport ATPase 2, *YDR*039C

*TSP*1	Synthase subunit of trehalose-6-phosphate synthase/phosphatase complex, which synthetizes the storage carbohydrate trehalose; also found in a monomeric form; expression in induced by the stress	*BYP*1,*CIF*1, *FDP*1, General glucose sensor subunit 1, *GGS*1,*GLC*6, Glycogen metabolism control protein *GLC*6, Trehalose-6-Phosphate synthase, Trehalose synthase complex catalytic subunit *TPS*1, *TSS*1, UDP-glucose-glucosephosphate glucosyltransferase, *YBR*0922, *YBR*126C
*CIN*5	Basic leucine zipper transcriptional factor of the type yAP-1 family that mediates pleiotropic drug resistance and salt tolerance; localizes constitutively to the nucleus	AP-1-like transcription factor *YAP*4, Chromosome instability protein 5, *HAL*6, *OR*26.18, *SDS* 15, Transcription activator *CIN*5, *YAP*4, *YOR*028C
*HSP*12	Plasma membrane localized protein that protects membranes from desiccation; indiced by heat shock oxidative stress, osmostress, stationary phase entry, glucose depletion,oleate and alcohol	12 kDa heat shock protein, *GLP*1, glucose and lipid regulated protein, *HOR*5, *YFL*014W
*CTT*1	Cytosolic catalase T, has a role in protection from oxidative damage by hydrogen peroxide	Catalase T, *YGR*088W
*MSN*2	Transcriptional activator related to Msn4p; activated in stress conditions, which results in translocation from the cytoplasm to the nucleus; binds DNA at stress response elements of responsive genes	Multicopy suppressor of *SNF*1 protein 2, *YM*9532.02C, *YMR*037C, Zinc finger protein *MSN*2
*MSN*1	Transcriptional activator involved in regulation of invertase and glycoamylase expression , invasive growth and pseudohyphal differentiation , iron uptake, chlorium accumulation, and response to osmotic stress	*FUP*1, *HRB*382, *MSS*10, Mylticopy suppressor of SNF1 protein 1, *PHD*2, protein *MSN*1, *YOL*116W
*HOR*2	One of two redundant DL-glycerol-3-Phosphatases (*RHR*2/*GPP*1 encodes the other) involved in glycerol biosynthesis;	DL-glycerol-3-phosphatase 2, *GPP*2, *YER*062C

*GPD*1	NAD-depended glycerol-3-phosphate dehydrogonase, key enzyme of glycerol synthesis, essential for growth under osmotic stress; expression regulated by high-osmolarity glycerol	D2830, *DAR*1, *HOR*1, *OSG*1, *OSR*5, *YDL*022W
*HOT*1	Transcription factor required for the transient induction of glycerol biosynthetic genes *GPD*1 and *GPP*2 in response to high osmolarity; targets Hog1p to osmostress responsive promoters	Hypothetical 79.4 kDa protein in *ALD*2-*DDR*48 intergenic region, *YM*8010.02, *YMR*172W
*SKO*1	Basic leucine zipper (bZIP)transcription factor of ATF/CREB family that forms a complex with Tup1p and Ssn6p to both activate and repress transcription; cytosolic and nuclear protein involved in osmotic stress	*ACR*1, CRE-binding bZIP protein *SKO*1, N1702, *YNL*167C
*HOG*1	Mitogen activated protein kinase involved in osmoregulation via three independent osmosensors; mediates the recruitment and activation of RNA Pol II at Hot1p-depended promoters	L2931, L9254.2, MAP kinase *HOG*1, Mitogen-activated protein Kinase *HOG*1, osmosensing protein *HOG*1, *SSK*3, *YLR*113W
*CYC*8	General transcriptional co-repressor, acts together with Tup1p; also acts as part of a transcriptional co-activator complex that recruits the SWI/SNF and SAGA complexes to promoters	*CRT*8. glucose repression mediator protein, *SSN*6, *YBR*0908, *YBR*112C
*HSP*104	Heat shock protein that cooperates with Ydj1p(Hsp40) and Ssa1p(Hsp70) to refold and reactivate previously denaturated, aggregated proteins; responsive	Heat shock protein 104, L0948,*YLL*026W

1.5 Aims and objectives of this research

The overall aim of this work was to elucidate some outstanding issues concerning yeast performance during fermentations for ethanol production. Specifically, work has focused on evaluation of NaCl-induced osmotic stress responses in industrial winemaking strains of the yeast, *Saccharomyces cerevisiae* and relationships with ethanol tolerance. The following parameters have been the subject of experimental research:

- Viability and growth rate in stressed yeast
- Yeast fermentation ability
- Yeast ethanol tolerance
- Yeast high sugar tolerance
- Yeast evaluation for product improvement

The work has primarily been conducted in the laboratories of the Technological Educational Institute of Athens (Greece) in collaboration with Professor Elias Nerantzis and in Georgakopoulos Estate winery.
Experiments for yeast growth, and viability of yeast cells under different concentrations of sodium chloride have been performed both at lab and industrial scale. Specifically, we investigated the effect of salt-stress on yeast growth, glucose consumption and yeast viability following salt-preconditioning in batch and continuous fermentation systems.
Subsequent fermentation with increased concentrations of glucose

using pre-conditioned cells has been performed to evaluate the alcohol tolerance of yeast and fermentation performance.

These were performed to evaluate the effect of osmotic stress on yeast ethanol tolerance and to sugar stress in relation to practical winemaking. This was deemed very important because the ability of yeasts to ferment high sugar concentration grape musts and to produce high levels of alcohol is linked with the production of specific kinds of wine like sweet wines and fortified wines.

2. MATERIALS AND METHODS

2.1 Laboratory scale experiments

2.1.1 Yeasts Cultures and Growth Conditions

Four different yeast strains of *Saccharomyces cerevisiae* and two non-*Saccharomyces* strains were used for laboratory experiments. *Vin 13* was kindly gifted by Anchor Ltd. (Capetown, S. Africa), *Chardonnay, KD* and *SCM,* which were produced by Martin Viallate, were kindly gifted by Ampeloiniki S.A. Thessaloniki Greece. *Kluyveromyces thermotelerans* and *Kluyveromyces marxianus* were supplied by the University of Abertay Dundee yeast culture collection.

Yeast cells were grown in defined medium containing (per litre deionised water): 100g D-glucose , 1g $K2HPO_4$, 1g $K_2H_2PO_4$, 0.2g $ZnSO_4$, 0.2g $MgSO_4$, 2g yeast extract and 2g NH_4SO_4. All the media components were purchased from Sigma Chemical Company.

2.1.2 Inoculum preparation

Cell rehydration: 1g dry weight of yeast was diluted in 100 ml of deionised water in an Erlenmeyer flask of 250ml volume at 30-35 Celsius, for 30 min. Inocula for experimental fermentations were prepared as follows: after 48h of pre-culturing, 10 ml was collected and centrifuged at 5000rpm for 15min. Cells were resuspended in

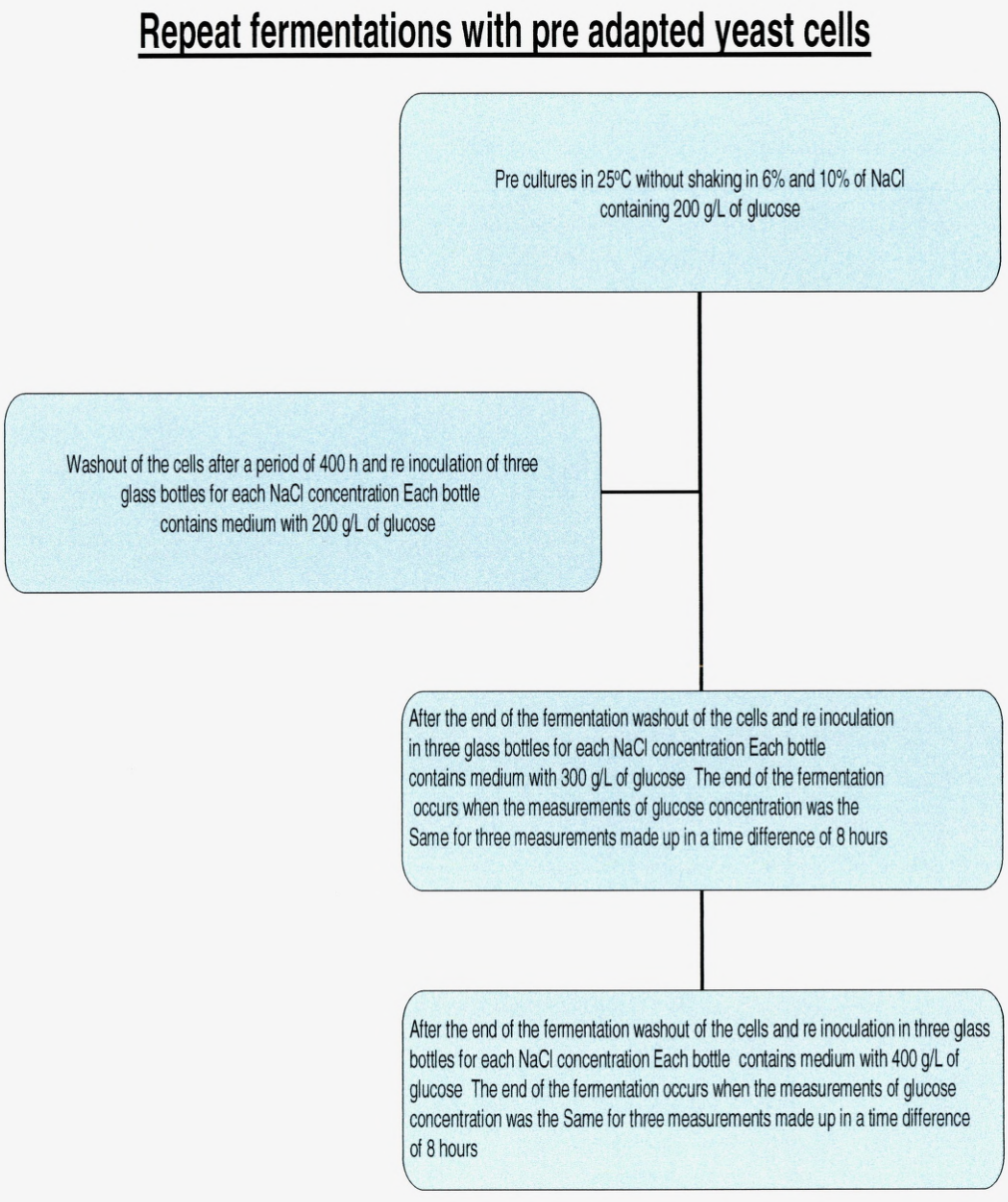
deionised water and re-centrifuged. This was repeated twice prior to determination of total cell number and cell viability (see 2.1.6) in the final washed inoculum. $5x10^5$ of living cells was used as inoculum to inoculate 250 ml of substrate.

2.1.3 Fermentation Media preparation

The medium for experimental laboratory fermentations consisted of the following: 200 g/L glucose, 1 g/L $K2HPO_4$, 1g/L $K_2H_2PO_4$, 0.2 g/L $ZnSO_4$, 0.2 g/L $MgSO_4$, 2g/L yeast extract and 2 g/L NH_4SO_4. Mineral components and the glucose were sterilised separately at 120°C , and 2Atm pressure for 20min. The pH was adjusted to 4 using 1N HCl solution. For salt stress induction experiments, medium contained NaCl (commercial NaCl was used) from 1 to 10% w/v and the total volume for the medium for each fermentation medium was 250 ml.

Batch fermentations were carried out in 300ml volume glass flasks containing 250ml of growth medium without shaking at 25°C. After inoculation 1mL were periodically taken direct from each flask in order to monitor the differences between stressed and un-stressed yeast cells with respect to yeast population growth and cell viability.

2.1.4 Repeat fermentations



Continuous (sequential) fermentations have been performed to evaluate the effect of NaCl to yeast fermentation performance under increased sugar concentrations. The following "salt pre-conditioning" was performed. Fermentation glass bottles (300ml capacity, each, 6

containing 250ml of growth medium) were prepared with 3 flasks containing 6% NaCl and 3 with 10% NaCl These 6 flasks had a concentration of D-glucose of 200g/L. Before each inoculation, yeast cells were centrifuged at 5000rpm for 10min and washed with 1% NaCl solution. This procedure was repeated at least 3 times before the cells were used as inoculum. After 400h fermentation, 6 additional flasks (3 flasks for each NaCl concentration) with a new medium containing 200g/L of D-glucose were inoculated with $5x10^5$ living cells pre-conditioned in 6% and 10% of NaCl as described above. After the end of the fermentation 6 additional flasks (3 flasks for NaCl concentration) each containing new medium of 250ml in volume with 300g/L of D-glucose were inoculated with $5x\ 10^5$ living cells. Finally, after the end of these fermentation 6 additional flasks (3 flasks for NaCl concentration) each containing new medium of 250ml in volume with 400g/L of D-glucose was inoculated with $5x10^5$ living cells. This experiment aimed to evaluate the tolerance of salt- preconditioned cells to ferment high sugar concentrations.
The end of the fermentation in each case occurred when the value of the residual sugars was the same for three continuous measurements over eight hour periods.

2.1.5 Yeast growth and viability determination

Yeast cell number was determined using a haemocytometer (Thoma type) and yeast cell viability using the methylene blue method of Lee et al., (1981). Yeast cell growth by colony counting was performed as

follows: Growth medium containing (g/L): 10 glucose, 5 peptone, 4 yeast extract and 15 agar was prepared. After sterilization at 120°C and 2Atm pressure for 20min, approximately 2mL of the medium was added to each Petri dish. Inoculation was made using 0.1mL from each fermentation flask. Serial dilutions for 0% NaCl were $1/10^{-5}$ and for 1, 2, 3, 4, 5% NaCl was $1/10^{-4}$. Three Petri dish spread plate dishes were used for each measurement.

Cell viability was determined using a haemocytometer (Thoma type). 1mL of sample medium was taken and diluted in 9 mL of deionised water. 1mL of this solution was dissolved with 1mL of 10% v/v methylene blue solution and left for 10 min. Aliquots of 1µL were placed on the haemocytometer by using a Pasteur pipette. The haemocytometer was then microscopically observed by an optical microscope (Olympus model CHK2-F-GS microscope). Yeast cell viability was calculated and expressed as follow: Viability (%) = a/n x 100

Where: a: number of metabolically active cells; n: total cell number. Since cellular viability needed to be determined immediately after hyperosmotic treatments, vital staining with methylene blue, which is rapid and accurate, was used. However, compared to methods that determine yeast reproducibility, methylene blue staining slightly overestimates cell viability (Jones, 1987). In this regard, comparison studies between methylene blue method and plate counting methods regarding yeast cell viability have been performed. The studies of viability in Petri dishes performed to check the accuracy of the methylene blue method regarding the hypothesis that NaCl effect

methylene blue and give faults results According to Figure 7 the difference between the two methods was under 5% and was 2.2%.

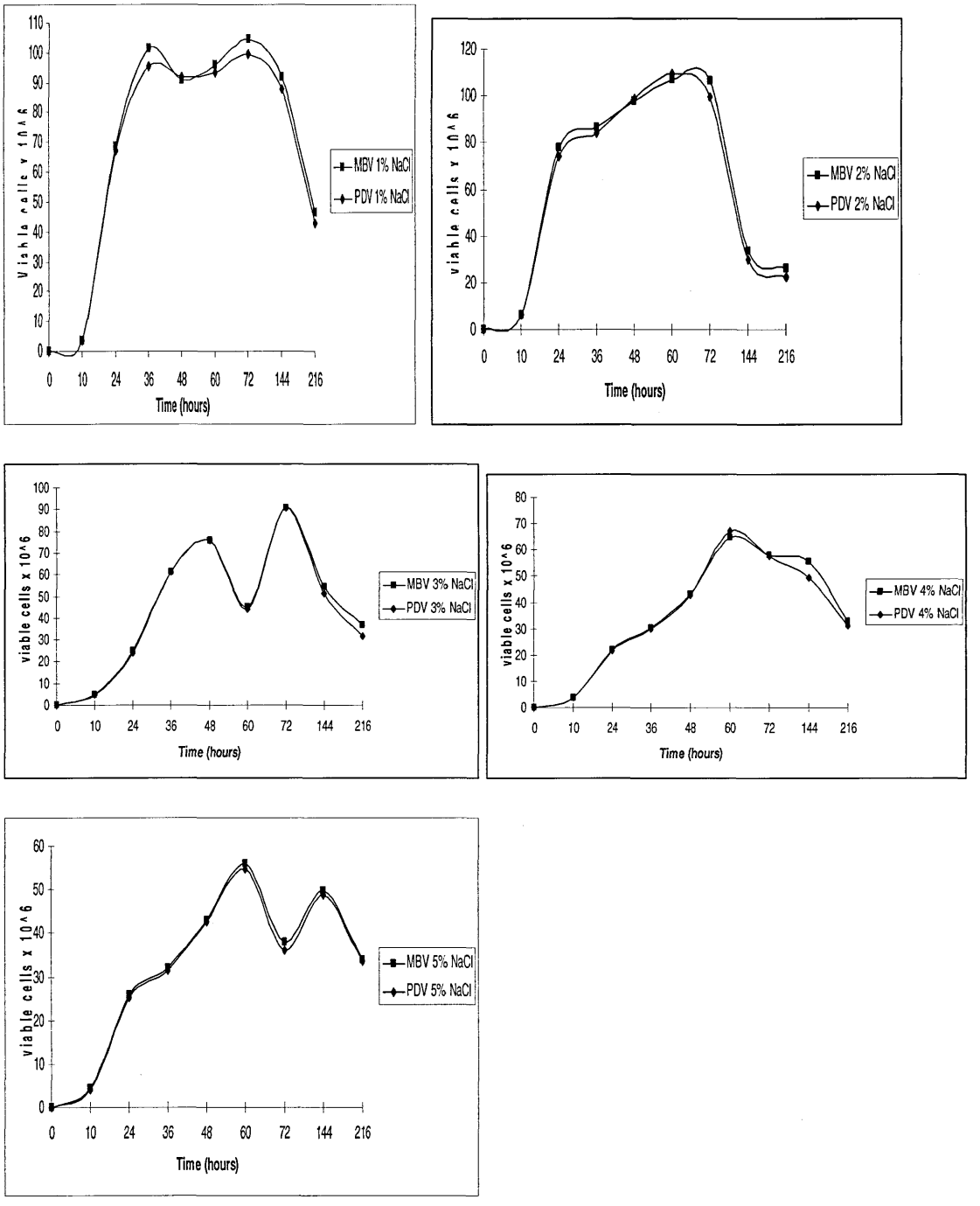


Figure 7 Comparison between methylene blue method (MBM) and Petri dishes method (PDM) for the measurement of the viable cells under osmotic stress produced by 1%-5% of NaCl.

Yeast mean cell volumes were determined using a Coulter Multisizer (Coulter Electronics, UK) according to the operators manual (at Abertay University, Dundee).

2.1.6 Glucose measurement

Glucose was determined using the DNS method (Miller 1959) for lab scale fermentations

2.1.7 Ethanol measurement

Ethanol was determined using an enzymatic kit from Boehringer Manheim/R-Biopharm Cat. No. 10 176 290 035. The principle of the measurement based on that:

Ethanol is oxidized to acetaldehyde by NAD in the presence of the enzyme alcohol dehydrogenase (ADH):

Ethanol + NAD -----) acetaldehyde + NADH + H^+

Acetaldehyde oxidized to acetic acid in the presence of aldehyde dehydrogenase (Al-DH)

Acetaldehyde + NAD^+ + H_2O -----) acetic acid + NADH + H^+

NADH is determined by means of its light absorption at 334, 340 or 365 nm.

2.1.8 Glycerol measurement

Glycerol was determined using an enzymatic kit from Boehringer Manheim/R-Biopharm Cat. No. 0148270. The principle of the

measurement based on that:

Glycerol is phosphorylated by ATP to L-glycerol-3-phosphate in the reaction catalyzed by glycerokinase (GK) :

Glycerol + ATP -----) L-glycerol-3-phosphate +ADP

The ADP formed in the above reaction is reconverted to ATP by phosphoenolopyruvate with the aid of pyruvate kinase:

ADP + PEP ------> ATP + pyruvate

In the presence of the enzyme L –lactate dehydrogenase (L –LDH) pyruvate is reduced to L-lactate by reduced nicotinamide-adendine dinucleotide (NADH) with the oxidation of NADH to NAD :

Pyruvate + NADH + H^+ ------> L-lactate + NAD

The amount of NADH oxidized in the above reaction is stoichiometric to the amount of glycerol. NADH is determined by means of its light absorption at 334, 340 or 365 nm.

2.1.9 Yeast Immobilization

Immobilization was carried out using a device described (as well as kindly offered) by Yokotsuka *et al.* (1997).

Double layer alginate beads were produced by simultaneous passing 2% sodium alginate solution through outer nozzle and sodium alginate and yeast suspension through inner nozzle with the aid of peristaltic pumps (Masterflex C/L models 77120-70 and 77120-62) into 0,1M $CaCl_2$ solution. Yeast gel suspension was produced by mixing equal volumes of 2% sodium alginate solution and yeast solution. Yeast cell concentration in gel suspension, expressed in cfu/mL, shown at the

following table:

Yeast = Vitilevure SCM	Y_0
Yeast cell concentration	$1.75x10^6$

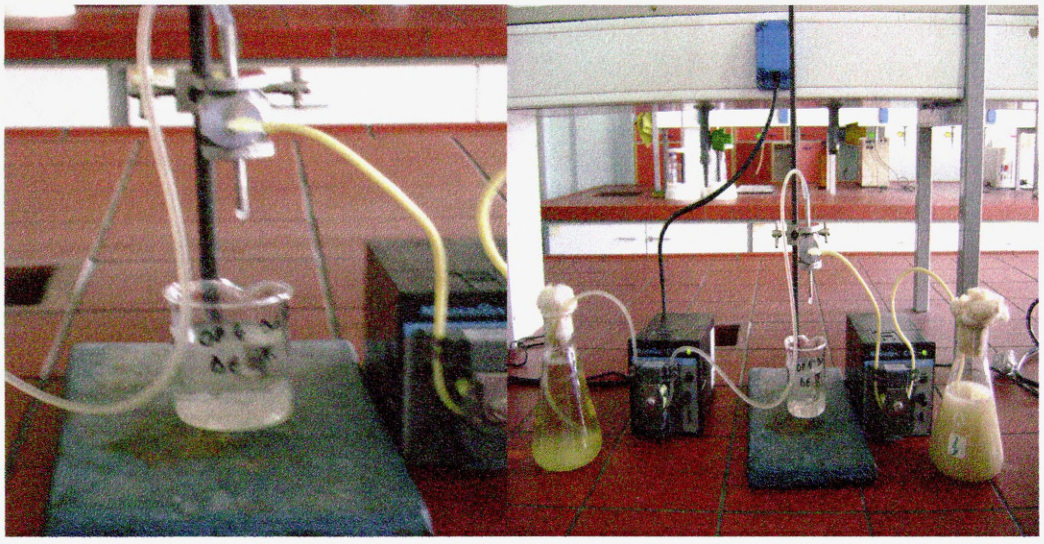


Figure 8 The Yokotsuka immobilization system

2.1.10 The WITY system

The WITY (the name of the system is the first letters from the words: Wine, Immobilization, Tower, Yeast) system that has been already described previously by Nerantzis, Logothetis and Loziou (1995) consists of one to four modular fermentation vessels. The vessels are designed to have a ratio of approx. 1:10 diameter to height aspect ratio. The ratio of volumetric capacity to total volume is 1:2. The vessels have an expansion at the top to help as a mechanical foam breaker. The fermentors are equipped with water jackets for temperature control. The modular fermentors have an inlet and outlet connected in series. The

outlet of the first is the inlet of the second and so on. The medium is introduced to the vessel with a peristaltic pump. The WITY system is shown in diagrammatic form in Fig 9 and in photograph in Fig 10.

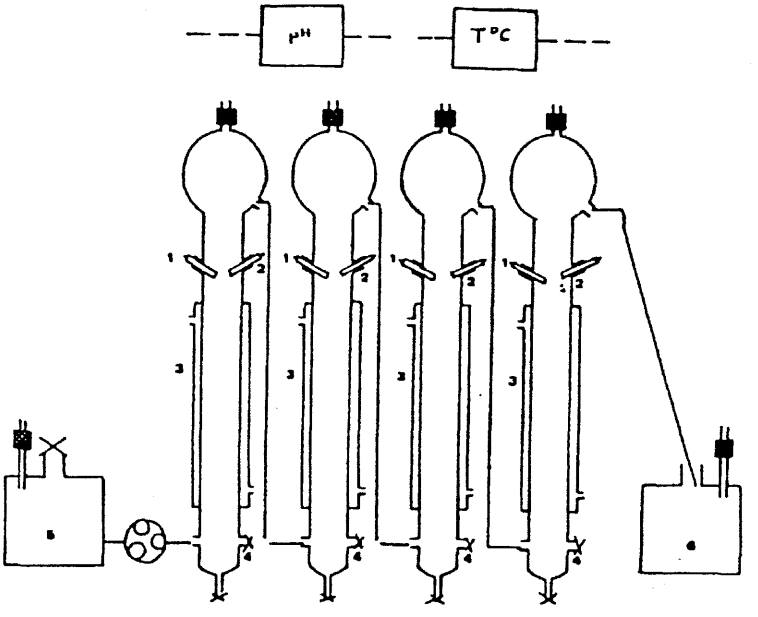


Figure 9. The modular tower fermentations system. The fermentors were filled with yeast cells double imrnobilized in alginate beats. The order in which the yeasts trains was from left to right 1: pH sensor, 2: temperature sensor,3 : water jacket ,4 :
Sampling port, 5: Feed vessel,6 : Product vessel.

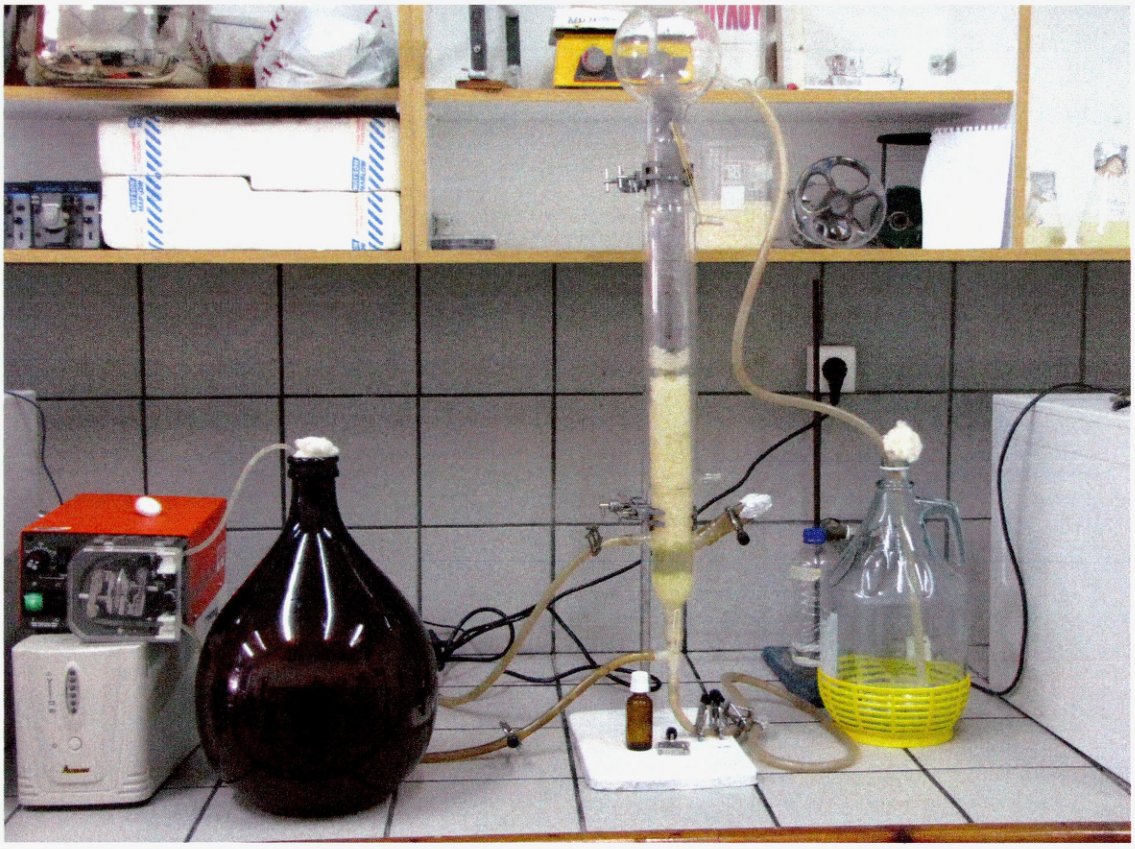


Figure 10 The tower fermentor

2.2 Industrial scale fermentations

For industrial scale fermentation experiments, a quantity of 40000L of must from Chardonnay and Merlot grape varieties was used over a four year harvest (2004-2008). Grapes cultivated at the same area of an altitude of 550 meters came from the same two hectares of vineyard. Cultivation methods were the same for each year and the grape harvest took place at the same period. A pre-fermentation procedure of cryo-extraction was made at the same temperature and for precisely twelve hours in each year of production except of the fourth year and for the Merlot grape variety. For inoculation to initiate

fermentation, different yeast strains for each year were employed and were kindly gifted from Martin Vialate. For the inoculum, 1250g of dry yeast preparation was diluted in 50L water containing 500g of sucrose at 35°C. Two inocula were made for each tank: one contained NaCl (6% w/v of commercial NaCl in crystal form was used) for yeast adaptation in osmotic conditions and the other without NaCl. After 16 hours inocula were added to each tank. Each fermentation tank was stainless steel, 5000L in volume (Figure 9). Fermentation temperature was maintained at 10 °C for all Chardonnay fermentations for the first three years but for the fourth year all fermentations as contacted at 15 oC. The analysis of industrial scale fermentations was made by FOSS Oenos WINE SCAN. This instrument can analyse and give 20 different parameters (ethanol, glycerol, volatile acidity, pH, total acidity, glucose, fructose, sucrose, acetic acid, tartartic acid, malic acid, galactic acid etc) using Fourier-transformed near infra red spectroscopy.

2.3 Statistical analyses

All experiments were conducted in triplicate and the results are presented as the average of three measurements with min and max standard error.

For each of the laboratory based experiments to examine either I: Effects of various salt concentrations on cell growth/viability II: effects of salt concentrations on different yeast species III: effect of salt preconditioning on fermentation performance three different fermentations were made. From each fermentation and every time three

samples were taken. Firstly, the average of the three values separately was calculated. Secondly, the average value of the three average values was calculated. The column of these results was analysed with the statistical programme called BioStat Plus 2008 version 5.3.0.6 by AnalySoft Comp using the Basic Describe Statistics package.

Figure 11 Fermentations tanks of 5000 L volume with temperature control system

3 RESULTS & DISCUSSION

3.1 The effect of NaCl on growth and viability of the industrial yeast strain, *S.cerevisiae VIN 13*

The relationship between microbial growth and viability and osmotic pressure has been a subject of research since the 1950's (Scott, 1956). The effects of sodium chloride and osmotic stress in general, on yeast physiology and metabolic ability have been studied widely, since the early 1980's (Esener *et al.*, 1981). When NaCl was added to a growth medium containing yeast cells the intracellular concentration of Na^+ increased, and reached a lower value than that of the medium. This increase of Na ions has a negative effect on yeast growth and viability (Rodriguez-Navarro and Ortega, 1981). Similarly, treatment of *S. cerevisiae* cells with KCl up to 6% caused a great loss of viability (Fuping *et al.*, 2005). As discussed previously, a gradual increase of sodium chloride in a growth medium containing yeast cells can cause a cell growth arrest depending on the sodium chloride concentration (Norberg and Blomberg, 1997). Experiments have shown that the leavening ability of bakers yeast decreases dramatically during cultivation in media containing sodium chloride concentrations between 0 and 3% (Oda and Tonomura, 1992). Prior research (Morris *et al.*, 1986) has shown that the greatest loss in cell viability was caused under hyper osmotic conditions between 0 and 1 OSM, (OSM= Osmolarity=5.85%NaCl w/v). Interestingly between 1 and 4 OSM the loss of viability was smaller (Morris *et al.*, 1986). Comparative studies

on the effect of osmotic stress in *S.cerevisiae* and non-*Saccharomyces* strains have shown that non-*Saccharomyces* strains displayed a better ability to grow in the presence of NaCl than *Saccharomyces* (Garsia *et al.*, 1997). In general, in all previous studies that have been made, osmotic stress and especially sodium chloride-induced stress, caused growth arrest and negatively affected the viability of yeast cells.

In the following laboratory-scale series of experiments (Figures 12 to 15) the effects of salt from concentrations ranging from 0-10% NaCl w/v on yeast (*S.cerevisiae VIN 13* strain) cell growth and viability were investigated.

Figure 12 demonstrates the effect of NaCl from 0 to 5% w/v and Figure 14 from 5 to 10% w/v of NaCl concentrations. These findings show that NaCl caused a growth arrest in yeast cells and the difference between untreated cells and the cells which were treated under the highest salt-induced osmotic stress (10% NaCl w/v) was around 2.5 x 10^6 cells. Actually, when sodium chloride concentration increased total cell number concomitantly decreased. It is important to recognise that the experiments employing 0 to 5% of NaCl were conducted in defined medium containing 100 g/L D-glucose. Results in Figure 11 show the beneficial effect of sodium chloride (from 5-10%w/v) on maintenance of yeast viability using defined medium with 200 g/L D-glucose. This latter glucose concentration was chosen as it reflects the situation in grape must where total sugar concentration is around 200 g/L. This, in turn, means that the final alcohol produced following fermentation will be around 12% v/v.

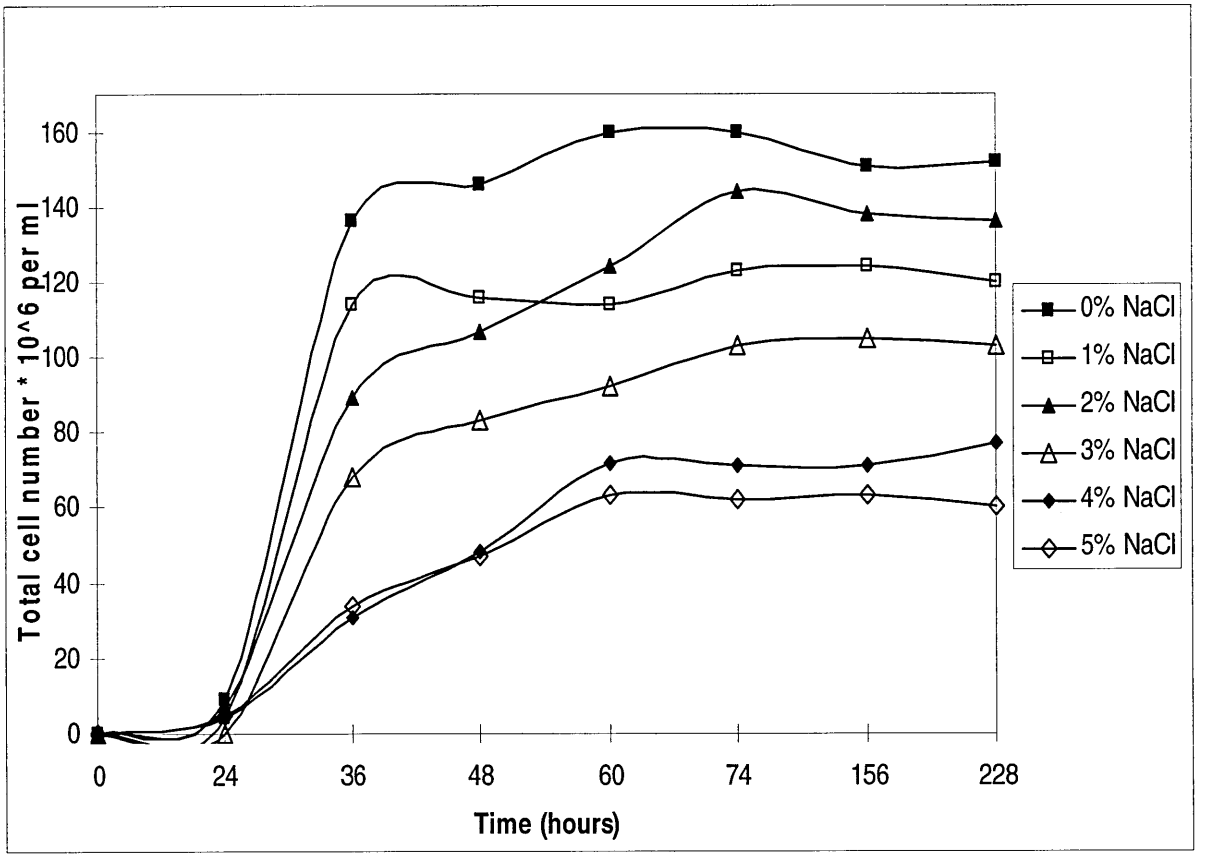


Figure 12 Influence of NaCl (0-5 % w/v) on yeast cell growth.

Saccharomyces cerevisiae (strain Vin 13 from Anchor, S.Africa) was grown in glucose-based (100g/L D-glucose) defined medium, without shaking at 25°C. Yeast cell number was determined using a haemocytometer at the intervals shown. Standard error was between 1.088 and 1.58%.

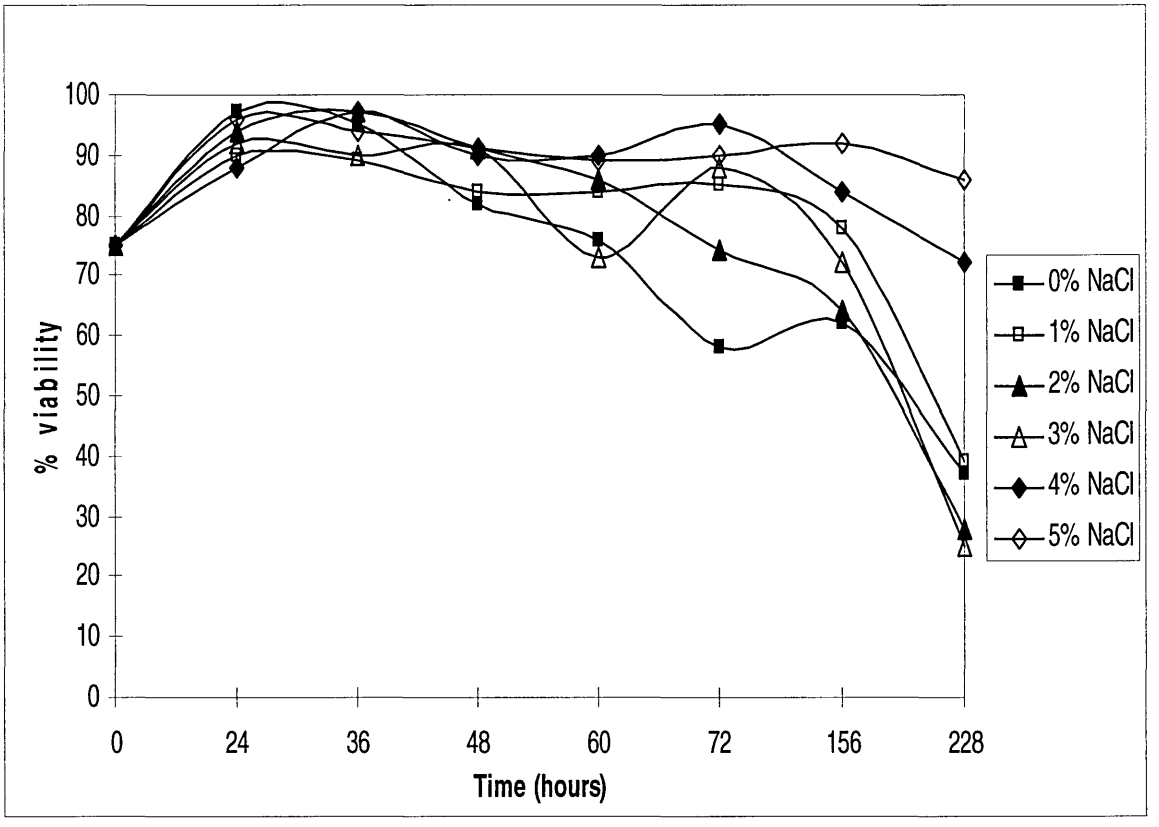


Figure 13 Influence of NaCl on yeast cell viability.

Saccharomyces cerevisiae (strain Vin 13) was grown in glucose-based (100 g/L D-glucose) defined medium, without shaking at 25°C. Yeast cell viability was determined by methylene blue staining using a haemocytometer at the intervals shown. Standard error was from 2.28 to 7.84%

Figures 13 and 15 show the effect of sodium chloride on yeast cell viability and the results demonstrate a positive affect on viability following treatment of cells under the highest salt-induced osmotic stress conditions.

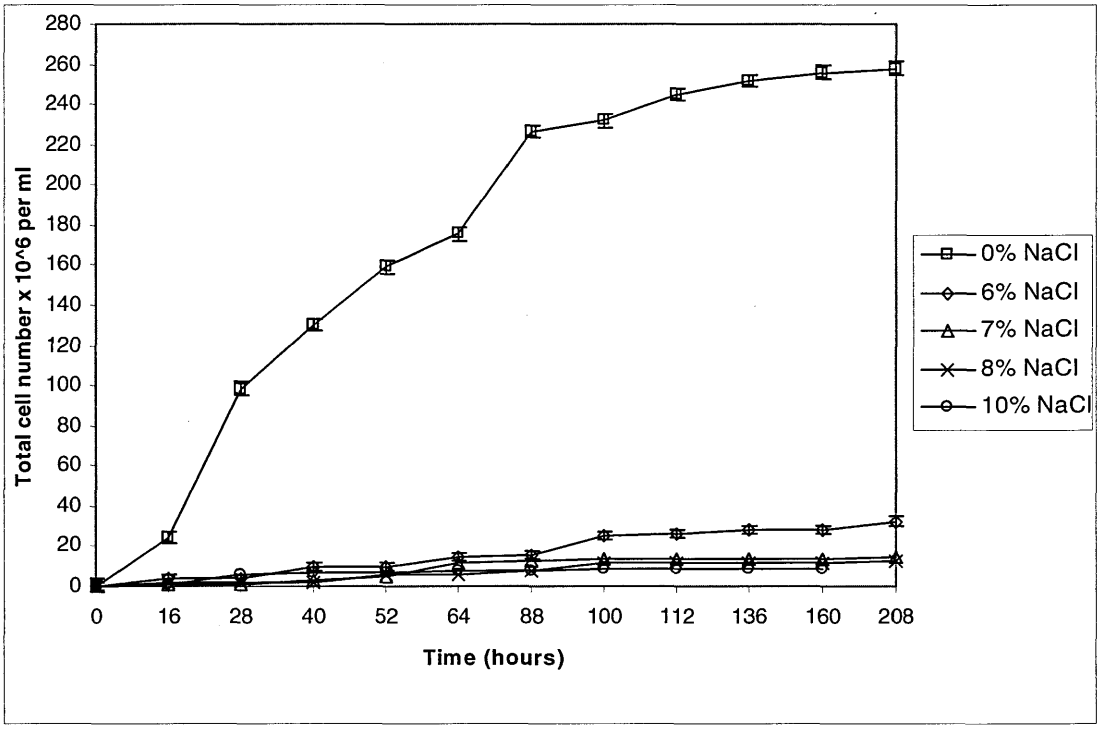


Figure 14 Influence of NaCl (0-10% w/v) on yeast cell growth.

Saccharomyces cerevisiae (strain Vin 13) was grown in glucose-based (200 g/L D-glucose) defined medium, without shaking at 25°C. Yeast cell number was determined using a haemocytometer at the intervals shown. Standard error was between 0.8 and 3.19%

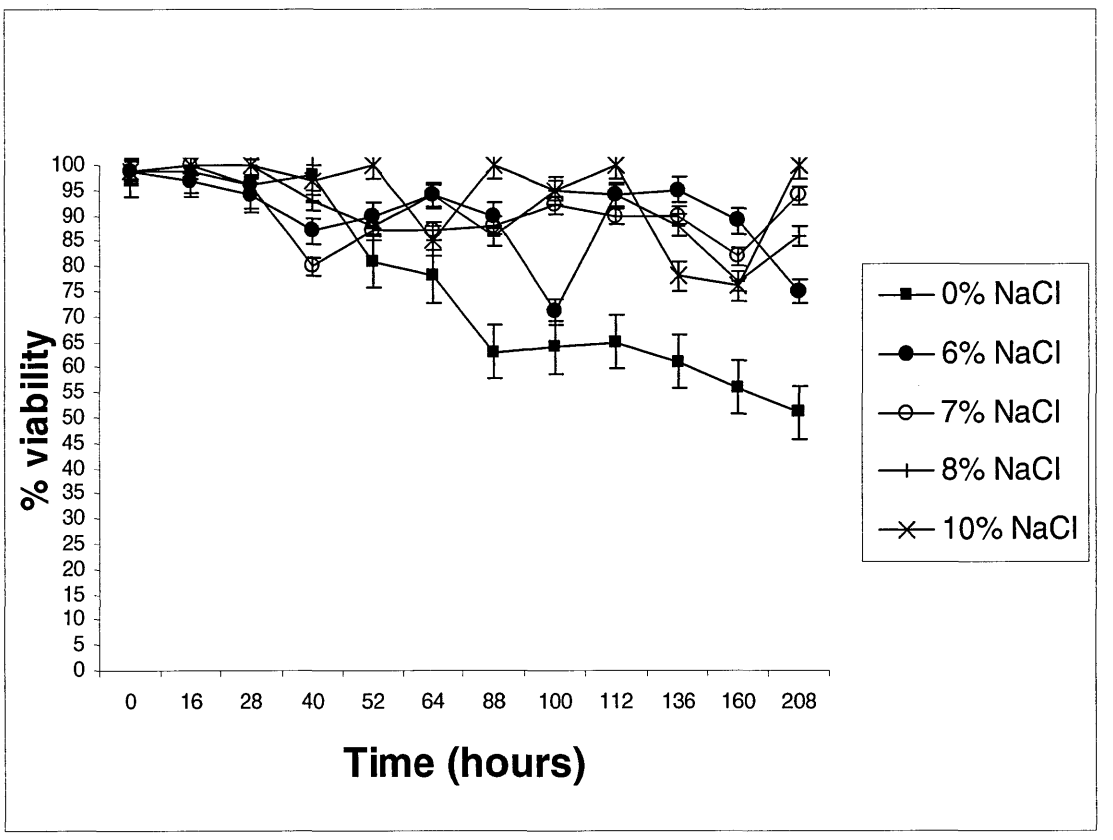


Figure 15 Influence of NaCl (0-10% w/v) on yeast cell viability.

Saccharomyces cerevisiae (strain Vin 13) was grown in glucose-based (200 g/L D-glucose) defined medium, without shaking at 25°C. Yeast cell viability was determined by methylene blue staining using a haemocytometer at the intervals shown. Standard error was between 1.75 and 5.28%

The results shown in Fig 12 and 14 are in accordance with previously published findings (Burg *et al.*, 2001; Hohmann and Magner,2005) in that NaCl-induced osmotic stress causes a growth arrest for yeasts cells. When osmotic stress (NaCl concentration) is gradually increased, a decrease in yeast growth and the total cell number during time occurred. However, regarding viability, cells treated under higher osmotic shock conditions (> 5% w/v NaCl) resulted in cells possessing a higher viability at the end of the fermentation (Fig 12 and 14). If we

compare Figs 12, 14 and Figs 13, 15 showing yeast growth and yeast viability, it can be seen that yeast cells exposed to higher concentrations of sodium chloride have the lowest growth but the highest viability compared with cells which were treated under lower osmotic shock conditions. Previous research supports the concept that when *S. cerevisiae* cells are exposed to high concentrations of NaCl, hey show reduced viability resulting in cells acquiring tolerance against a severe salt shock (up to 1.4 M NaCl= 8.19 % NaCl w/v) following a previous treatment with 0.7 M NaCl (4.09 % NaCl w/v) (Varela *et al.*, 1992). Overall, previous reports describe the osmotic stress of yeast cells for no more than a few hours, but in the current research cells were treated for at least 228 hours (Fig 12) during the fermentation process. In Figs 12 to 15 the results are contrary to published findings, especially concerning yeast cell viability (Figs 13 and 15).

Previous studies have reported that some agents, like sodium chloride, play an important role in minimizing or inhibiting the fermentation process, specifically with regard to glucose utilization for the production of biomass. (El-Samargy and Zall, 1988). Much earlier studies regarding this subject have been performed. For example, in the early 20's, studies concerned with the fermentability of yeasts during cereal wort fermentation under different sodium chloride concentrations showed that the fermentative ability of yeast increased when pre-conditioned with 5% w/v NaCl, but for higher concentrations, a gradual diminishing fermentative efficiency was reported (Speakman *et*

al.,1928). In the present work, it is clearly shown that for concentrations from 1 to 5% w/v NaCl, no difference in glucose utilization occurred and the fermentability of the yeast cells was approximately the same for all salt concentrations tested (Figure 16).

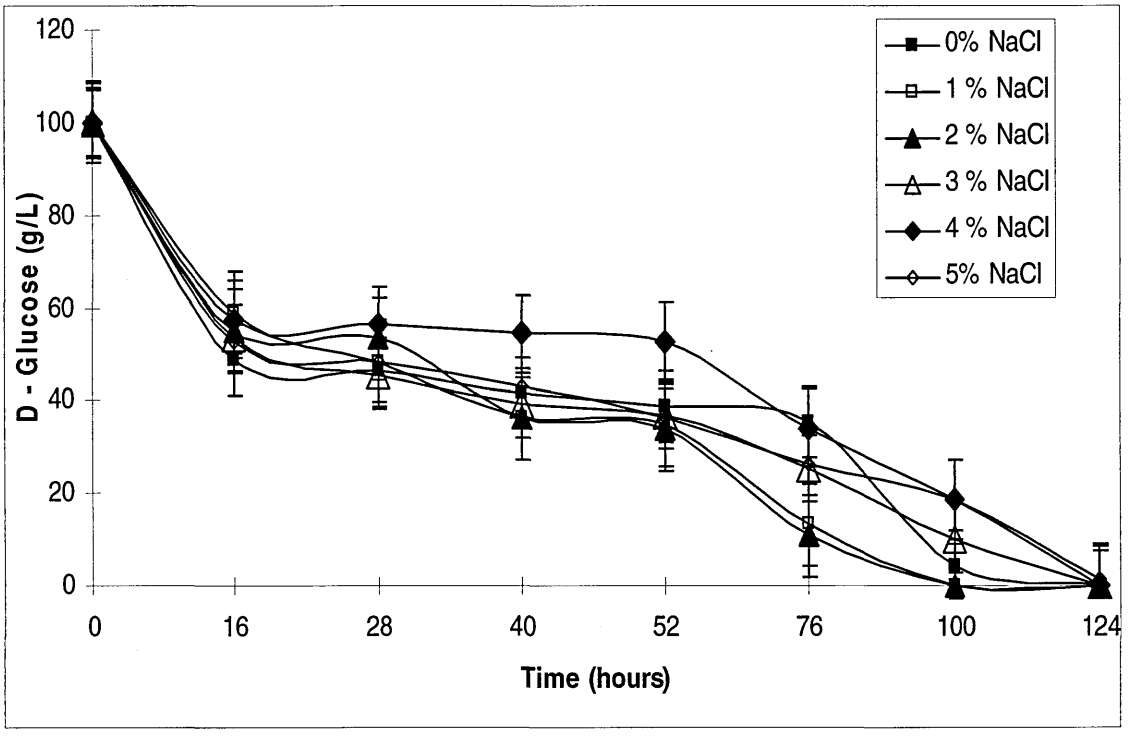


Figure 16 Influence of salt-induced osmotic stress (0-5% NaCl) on sugar consumption by yeasts.
Saccharomyces cerevisiae (strain Vin 13) was grown in glucose-based (100 g/L D-glucose) defined medium, without shaking at 25°C containing 100 g/L of D-glucose. Sugar consumption was determined by DNS method and the standard error was between 7.29 and 8.88 %.

Figures 17-20 show a comparative affect of sodium chloride on yeast growth, viability and sugar consumption when cells were treated with NaCl (range between 0% and 10% w/v). Additionally, Table 2 shows calculations of specific growth rate (μ) of osmotically stressed cells.

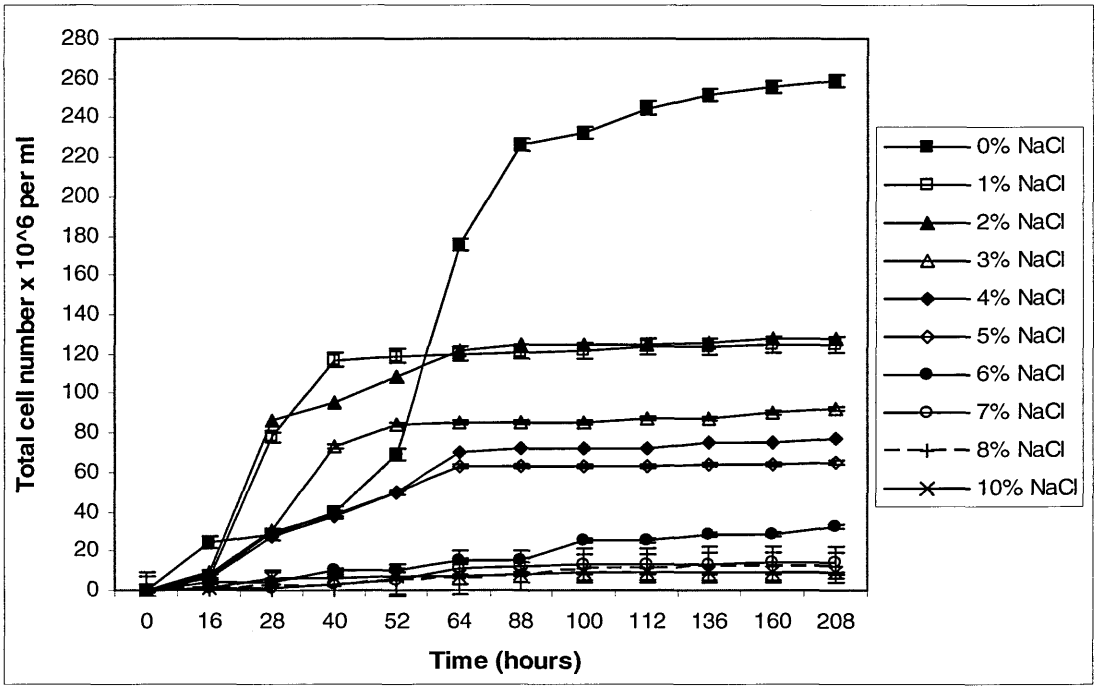


Figure 17 Influence of salt-induced osmotic stress (0-10% NaCl) on yeast cell growth.
Saccharomyces cerevisiae (strain Vin 13) was grown in glucose-based (200 g/L D-glucose) defined medium, without shaking at 25°C. Yeast cell growth was determined using a haemocytometer at the intervals shown. Standard error was between 1.30 and 8.28%.

From Fig 17 it can be seen that higher sodium chloride concentrations have a suppressive effect on yeast growth. The difference between total cell number of stressed cells and non stressed cells is almost 2.5 million cells/mL after 208 hours.

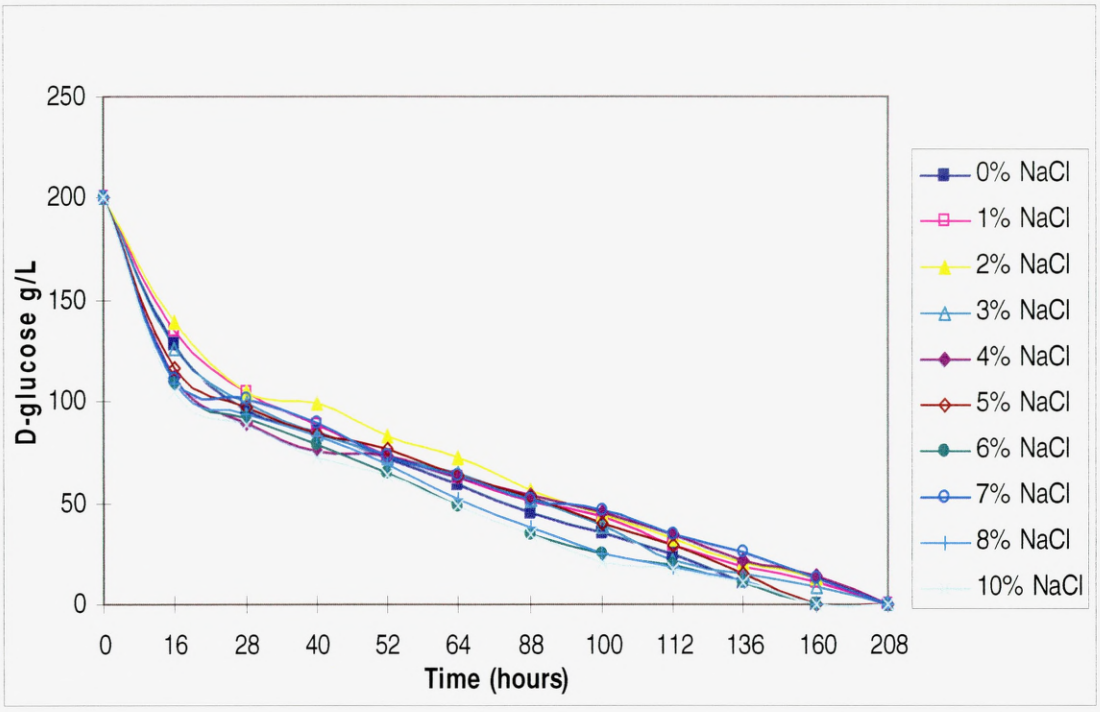


Figure 18 Influence of salt-induced osmotic stress (0-10% w/v NaCl) on sugar consumption.
Saccharomyces cerevisiae (strain Vin 13) was grown in glucose-based(200 g/L D-glucose) defined medium, without shaking at 25°C containing 200 g/L of D-Glucose. Sugar consumption was determined by the DNS method and the standard error was between 1.09 and 3.03%.

Interestingly, sugar consumption was unaffected by osmotic stress and, in fact, residual glucose reached very low levels for all NaCl concentrations tested. This phenomenon was previously observed for sodium chloride concentrations from 1% to 5% w/v and for sugar concentrations at 100 g/L. Using the data from Fig 16 it was possible to calculate the effect of osmotic stress on μ (specific growth rate). See Table 2.

Table 2 Calculation of μ for salt-stressed yeast cells. Where is μ = ln(x)-ln(xo)/t

time(hours)	0%	1%	2%	3%	4%	5%	6%	7%	8%	10%
0	0	0	0	0	0	0	0	0	0	0
16	0.27	0.18	0.21	0,2	0.18	0.19	0.15	0.09	0.10	0.07
28	0.16	0.19	0.20	0.16	0.16	0.16	0.09	0.05	0.06	0.10
40	0.12	0.14	0.14	0.13	0.12	0.12	0.08	0.05	0.05	0.07
52	0.10	0.11	0.11	0.10	0.09	0.09	0.06	0.05	0.05	0.05
64	0.09	0.09	0.09	0.08	0.08	0.08	0.06	0.05	0.04	0.05
88	0.07	0.06	0.06	0.06	0.06	0.06	0.04	0.04	0.03	0.03
100	0.06	0.06	0.06	0.05	0.05	0.05	0.04	0.03	0.03	0.03
112	0.05	0.05	0.05	0.05	0.04	0.04	0.03	0.03	0.03	0.03
136	0.04	0.04	0.04	0.04	0.04	0.03	0.03	0.02	0.02	0.02
160	0.04	0.03	0.03	0.03	0.03	0.03	0.02	0.02	0.02	0.02
208	0.03	0.02	0.02	0.02	0.02	0.02	0.02	0.01	0.01	0.01

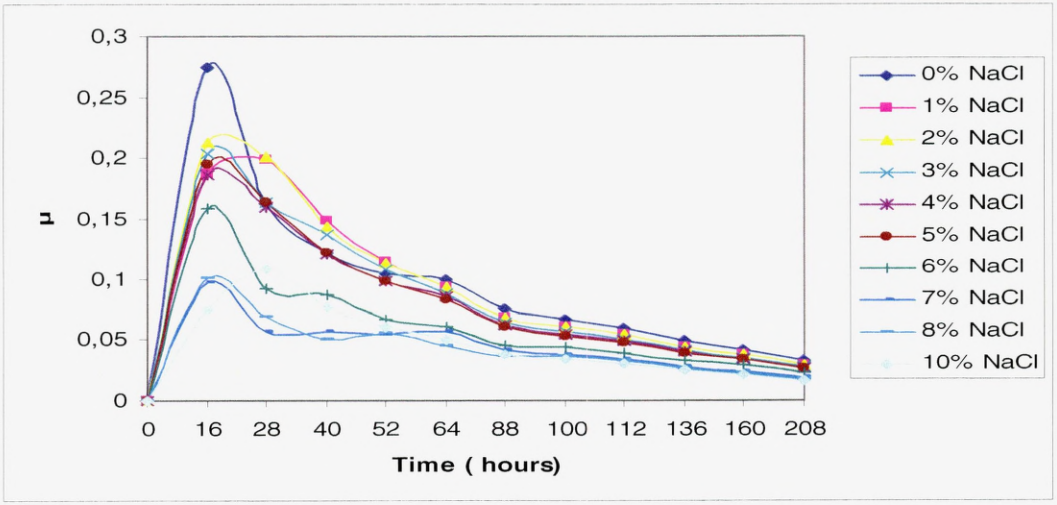
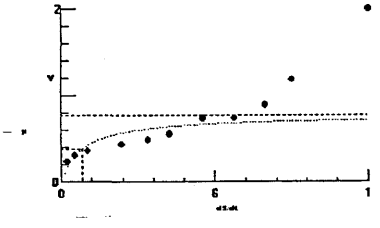


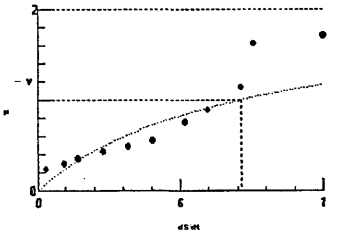
Figure 19 Influence of salt-induced osmotic stress (0-10% w/v NaCl) on μ *Saccharomyces cerevisiae* (strain Vin 13) was grown in glucose-based (200 g/L D-glucose) defined medium, without shaking at 25°C containing 200 g/L of D-Glucose

Like growth, the specific growth rate (μ) declines when NaCl concentration increases. The maximum rate appears at 16 hours for 0%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, and for 1% and for 10% w/v of NaCl appears at 28 hours. This means that sodium chloride has similar effects on specific growth rate as with net cell growth. The relationship between μ (specific growth rate) and the sugar consumption (dS) rate for different NaCl concentrations was extrapolated using a hyperbolic regression software package (J.S. Easterby) available online and represents a Michaelis-Menten type relationship (see Fig 20).

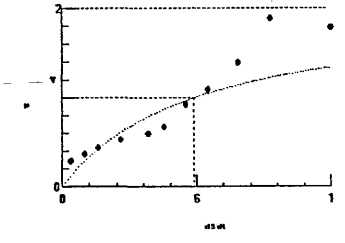
A) 0% NaCl



C) 2% NaCl



B) 1% NaCl



D) 3% NaCl

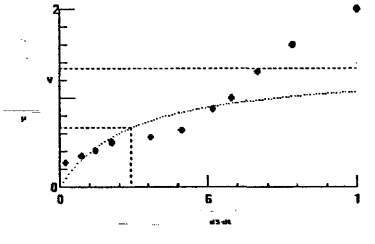
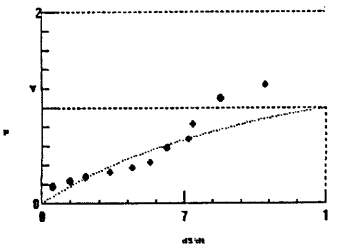
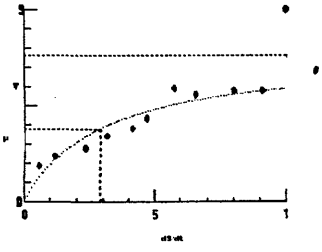


Figure 20a Michaelis-Menten type hyperbolic graphs for yeast (Vin 13) cultivated under osmotic stress conditions Where A: 0% NaCl w/v, B:1% NaCl w/v, C:2% NaCl w/v, D:3% NaCl w/v

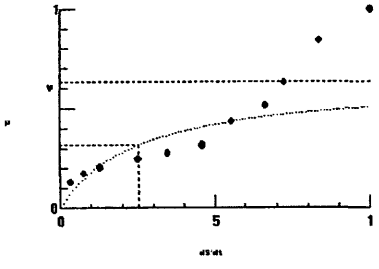
E) 4% NaCl



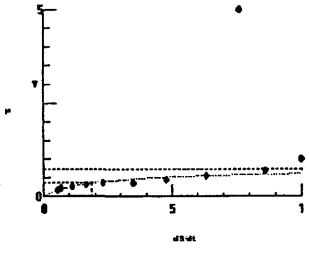
H) 7% NaCl



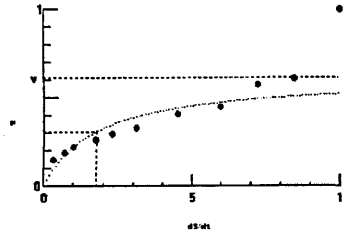
F) 5% NaCl



I) 8% NaCl



G) 6% NaCl



J) 10% NaCl

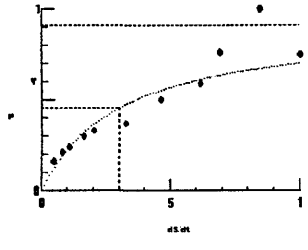


Figure 20b Michaelis-Menten type hyperbolic graphs for yeast (Vin 13) cultivated under osmotic stress conditions Where, E:4% NaCl w/v, F:5% NaCl w/v, G:6% NaCl w/v, H:7% NaCl w/v, I: 8% NaCl w/v, J:10% NaCl w/v

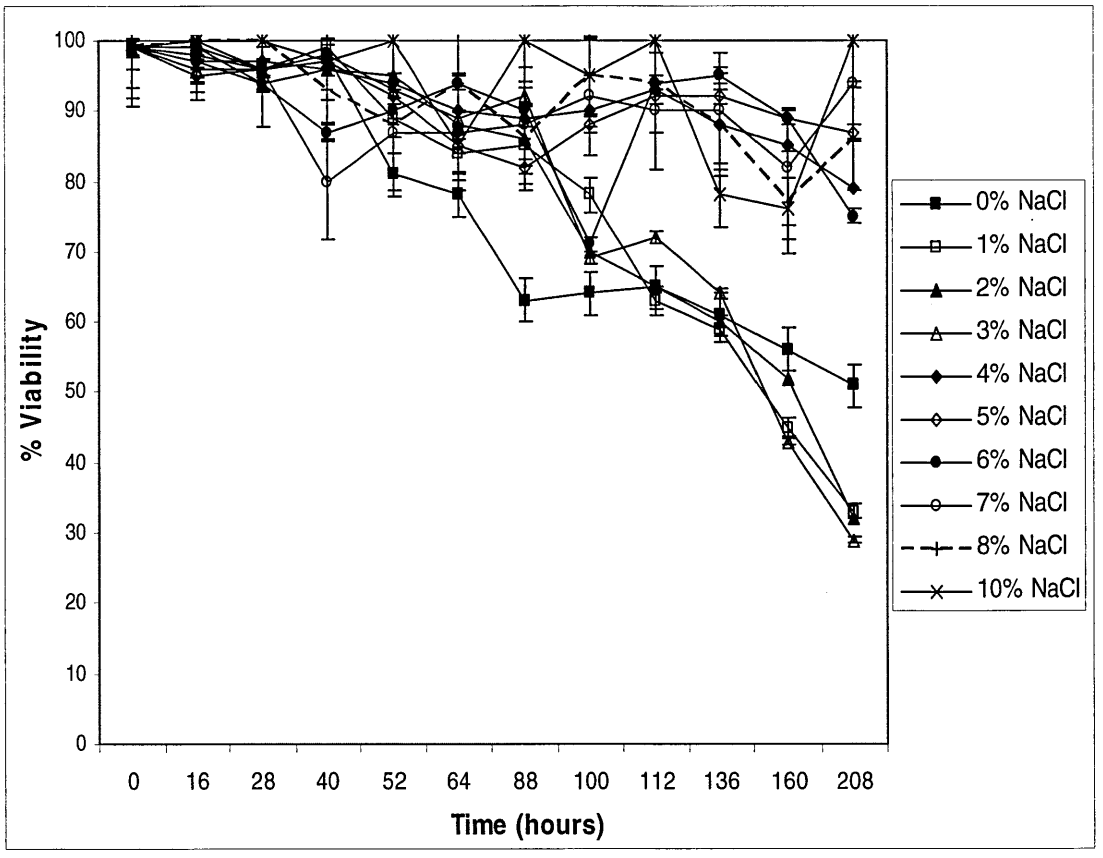


Figure 21 Influence of salt-induced osmotic stress (0-10% w/v NaCl) on yeast cell viability.
Saccharomyces cerevisiae (strain Vin 13) was grown in glucose-based defined medium, without shaking at 25°C. Yeast cell viability was determined by methylene blue staining using a haemocytometer at the intervals shown and the standard error was between 1.75 and 5.28%.

Regarding cell viability, high resistance of the cells to salt stress conditions were observed following a range of NaCl treatments. Results showed that after 64h of fermentation, yeast viability for untreated cells and for low sodium chloride concentrations started to decrease but the viability for stressed cells remained high. After a period of 208h, viability for untreated cells fell to around 50% and cells under highest

osmotic conditions retained high viability. This may be due to the fact that under osmotic stress, and especially under salinity conditions, an increase in cellular electrolytes and a decrease in cellular water potential occurred (Soveral *et al.*, 2007; Shimizu *et al.*,2006). Consequently, rapid efflux of water, cytoskeletal collapse, intracellular damage and growth arrest, are the phenomena which follow the saline stress. Adaptation to these conditions by yeast cells include: retainment of turgor, polarized cytoskeleton, cellular damage repair and resumption of growth. It is conceivable that the response is controlled by the HOG MAP kinase pathway (see section 1.4). It has been reported that accumulation of glycerol, which is the main compatible solute that cells produce intracellularly to adapt to the differential extra and intracellular osmotic pressure, is strongly affected by growth temperature and causes the over expression of GPD 1and FPS1, which encodes the glycerol transport facilitator and glycerol-3- phosphate dehydrogenase (Wojda *et al.*, 2003). The temperature (24°C) in these published studies was very close to the temperature that we have run our experiments.

It has been reported that osmotic stress caused by 0.3M (1,75 % NaCl w/v) sodium chloride and for a time period of 1h may prolong the life span of yeasts (Swiecilo *et al*, 2000). The relationship between temperature and osmotic stress regarding osmotic tolerance of cells and viability has previously been reported. Under osmotic pressure of 49.2MPa cell viability was close to 94% at a temperature of 23°C, but under higher osmotic pressure of 99MPa the viability decreased

dramatically to 25%. Beney *et al* (2001) have reported that under the same conditions of high osmotic pressure of 99MPa, but at a temperature of 5°C, the viability remained at a high level of 81%.

Three sodium chloride concentrations (i.e. 4, 6 and 10% w/v) were chosen for further experimentation regarding the viability of cells treated under such osmotic stress conditions for an extended period of time after cessation of fermentation. These three concentrations of sodium chloride were: 4% NaCl w/v because it is the highest concentration that researchers have used until now, 6% because it was the concentration which demonstrated the best curve (see Fig 14) of the sodium chloride concentrations between 4% and 10% of NaCl regarding growth and viability and 10% NaCl because it was the concentration with the lowest growth but with the maximum viability and represents the highest concentration of NaCl on this experimental series.

Figures 22 to 25 represent the affect of NaCl (4, 6, 10% w/v) on yeast growth and viability. In particular, Fig 24 shows the affect of osmotic stress on yeast cell viability after the end of the fermentation and for a time period of 400 hours. In this figure we can see a positive affect since the viability of untreated cells was approximately 20% and for the salt-treated (10% NaCl) cells was approximately 70%. In Figure 25 we can clearly see the difference on yeast cell viability during the whole fermentation process and for a time period of 216 hours.

As all experiments in Section 3.1 were conducted with a single yeast strain (S. cerevisiae VIN13), it was decided to carry out research (especially on salt–related phenomena) on other *Saccharomyces* and

non-*Saccharomyces* strains of relevance in winemaking. These experiments are described in section 3.2.

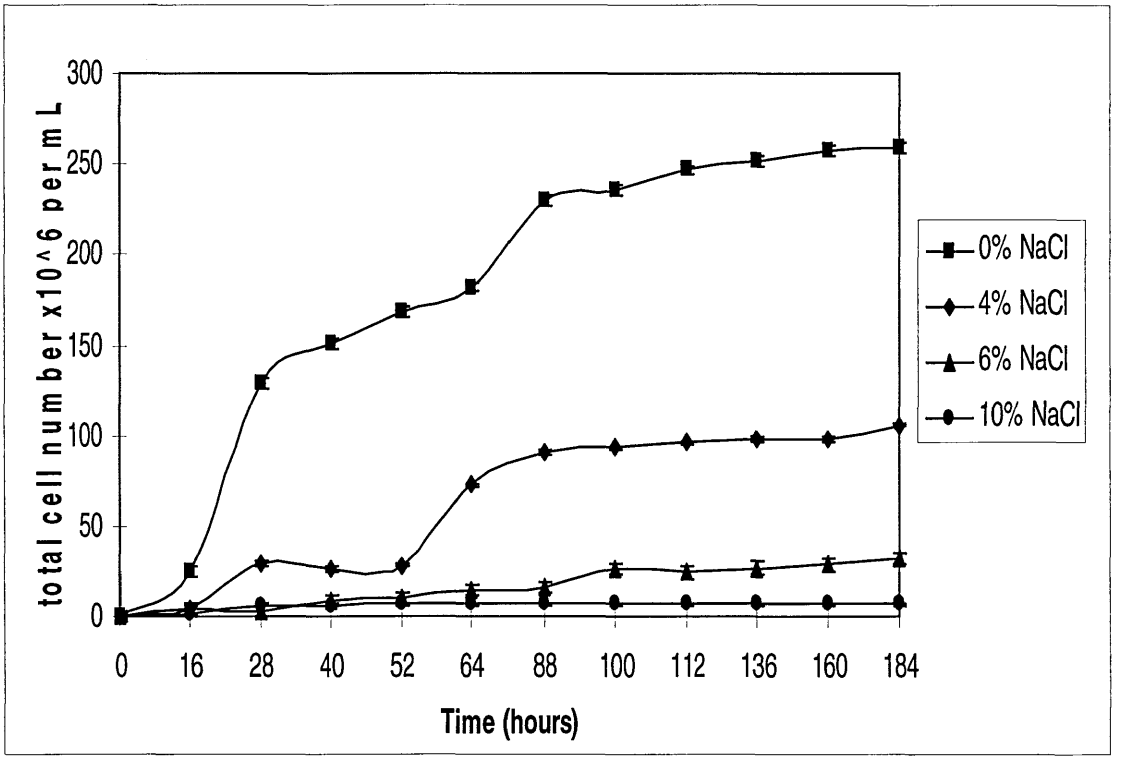


Figure 22 Influence of NaCl (0%, 4%, 6%, 10% NaCl w/v) on yeast cell growth.
Saccharomyces cerevisiae (strain Vin 13) was grown in glucose-based (200 g/L D-glucose) defined medium, without shaking at 25°C. Yeast cell growth was determined using haemocytometer and methylene blue method at the intervals shown. Standard error was from 0.65 to 2.57

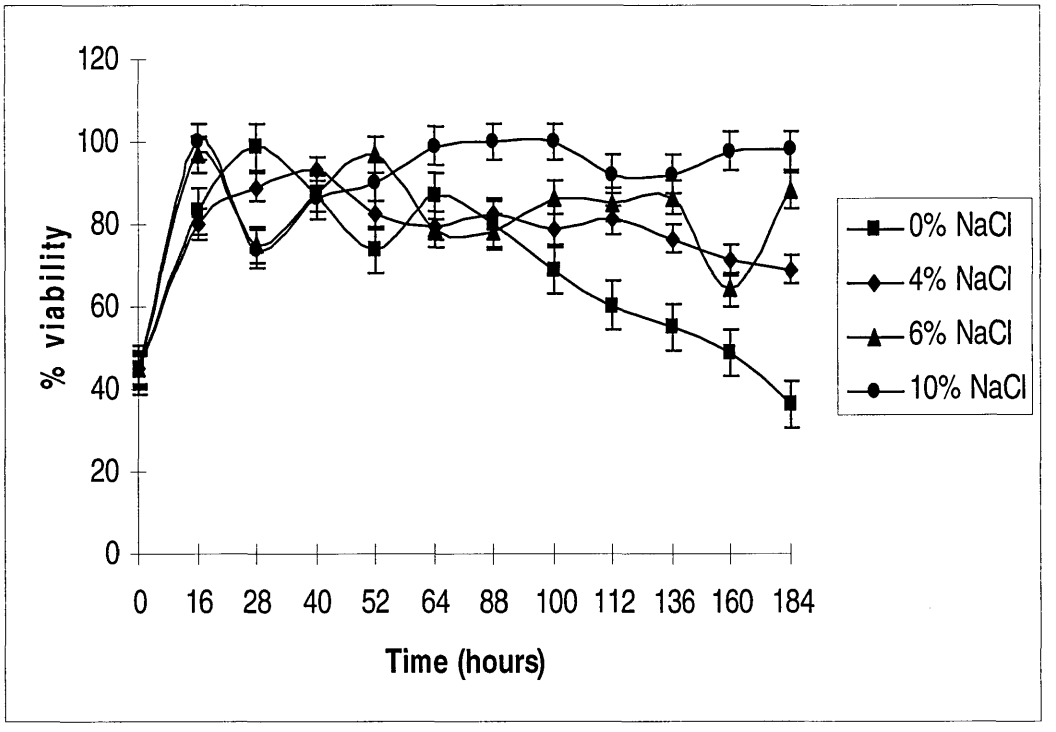


Figure 23 Influence of NaCl (0%, 4%, 6%, 10% NaCl w/v) on yeast cell viability
Saccharomyces cerevisiae (strain Vin 13 from Anchor, S.Africa) was grown in glucose-based (200 g/L D-glucose) defined medium, without shaking at 25°C. Yeast cell viability was determined using haemocytometer (Thoma type) and methylene blue method at the intervals shown. Standard error was from 1.75 to 5.28.

Figures 23 and 24 show that sodium chloride had a positive effect in maintaining yeast cell viability especially for extended fermentation periods of time. After 400h, cells treated with 6% and 10% NaCl had the highest viability. It is possible that maintenance of high yeast viability in the presence of high salt, was due to the synthesis of stress responsive compounds including trehalose and glycerol (see sections1.3.2 and 1.3.3).

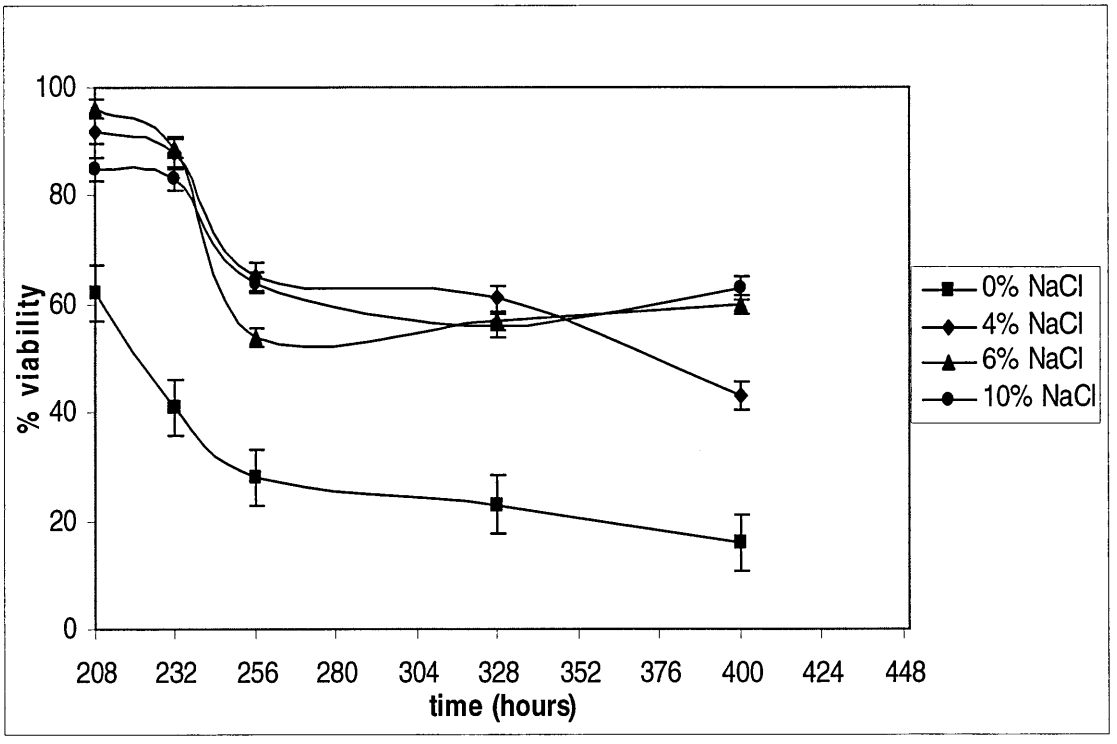


Figure 24. Yeast cell viability after fermentation under salt (0%, 4%, 6%, 10% NaCl w/v) stress
Cell viability over an extended period of 192 hours after fermentation for cells with 0% 4%, 6%, 10% w/v NaCl culture in defined medium (200 g/L D-glucose) without shaking at 25°C Yeast cell viability was determined using haemocytometer (Thoma type)and methylene blue method at the intervals shown. Standard error was from 1.75 to 5.28

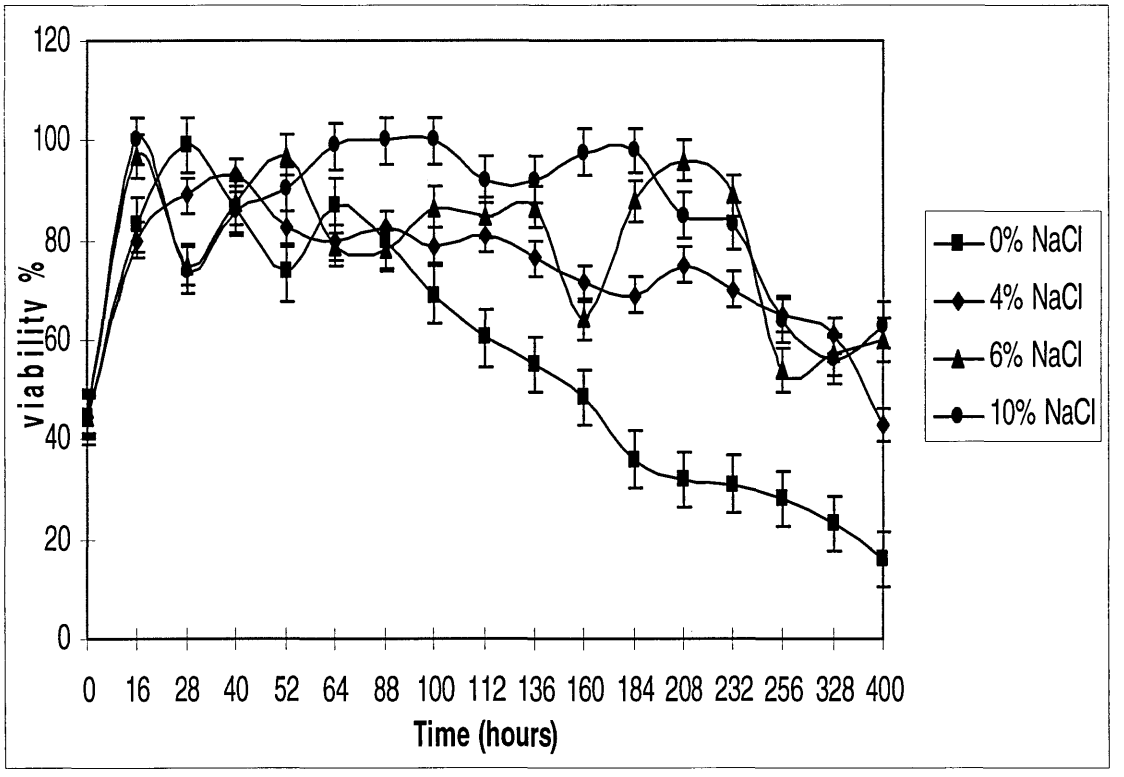


Figure25. Cell viability under salt stress (0-10%NaCl)
Cell viability over an extended time period of 400 hours including fermentation for cells with 0%, 4%, 6%, 10% w/v NaCl stress culture in defined medium (200 g/L D-glucose) without shaking at 25°C Standard error was from 3.51 to 5.66

In an attempt to investigate the affect of salt treatment on the long-term survival of yeast cells, we incubated cells for an extended time period of 645 days (~2 years). Figure 26 shows the results of viability maintenance in the presence of 6 or 10% w/v NaCl over this long-term yeast storage condition.

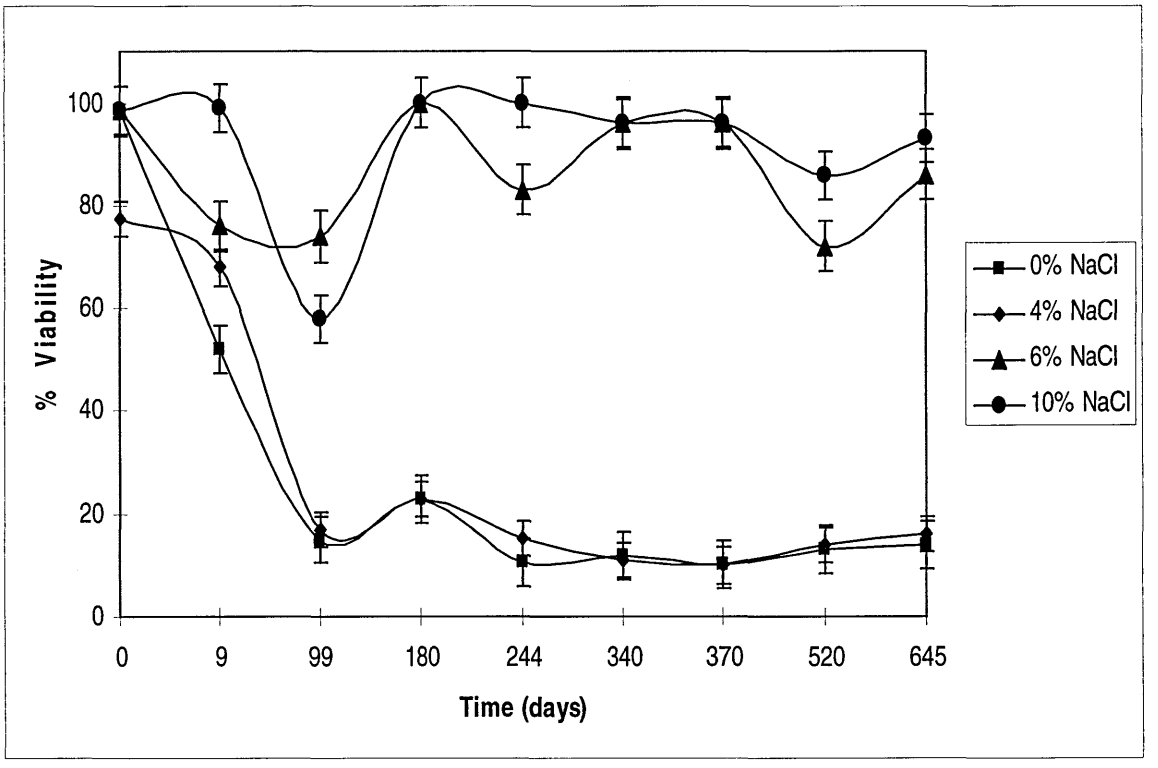


Figure26. Cell viability under salt stress for an extended time period of time

Cell viability over an extended time period of 645 days (including fermentation) for cells treated with 0%, 4%, 6%, 10% w/v NaCl in defined medium without shaking at 25°C.Standard error was from 5.28 to 5.75.

It is evident that treatment of cells for long time period has a positive affect for cells which were treated under the higher concentrations of NaCl (6 and 10% w/v NaCl). The difference between those cells and the cells which are treated with 4% w/v NaCl and without NaCl was approximately 60%, and representative micrographs of such cells are shown in Fig 27.

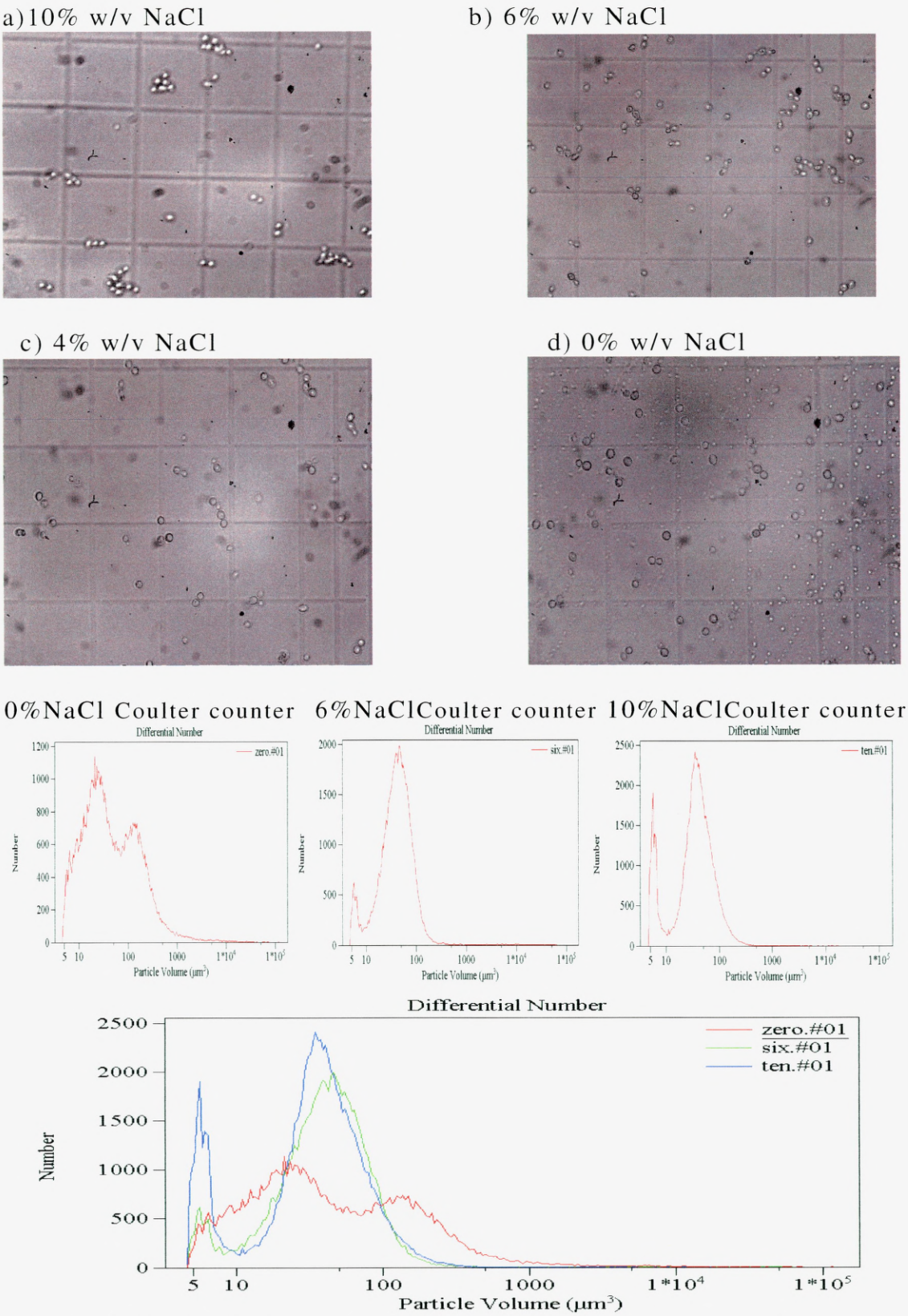


Fig 27 Cell viabilities and mean cell volumes of *S. cerevisiae* (strain VIN13) in long-term storage with and without salt treatments. Micrographs show methylene blue stained cells (Magnitude x 40), and the Coulter counter figures show mean cell volumes.

3.2 The effect of NaCl on growth and viability of three wine yeast strains of *S. cerevisiae* and in two non- *Saccharomyces* yeasts strains

Experimental fermentations have been conducted with three industrial yeast strains: *S. cerevisiae* Chardonnay, *S. cerevisiae* CSM and *S. cerevisiae* DK (which are wine yeasts used in the wine industry) and two non-*Saccharomyces* strains *K. thermotolerans* and *K. marxianus* (which exist as grape must micro flora) to investigate if similar phenomena occurred that found on *S.cerevisiae* strain VIN13 (section 3.1). Such yeasts were chosen since it is well known that during industrial wine making, alcoholic fermentation starts by non *Saccharomyces* species like *Kloeckera* or *Hansensiaspora.* Then a microbial succession occurs followed by *Saccharomyces* species and species like *Kluyveromyces, Tolulospora*, *Candida* and *Metchenikowia* (Fleet *et al*, 1984; Heard and Fleet, 1986; Ciani and Maccarelli 1998). *K. thermotolerans* and *K. marxianus* have been already used in industry. For example, *K. thermotolerans* has been employed in mixed culture with *Saccharomyces cerevisiae* by C. Hansen Ltd (Copenhagen) wine fermentation industries. *K. thermotolerans* has been a subject of research as a fermentation *multistarter* culture for alcoholic fermentations (Ciani *et al*, 2004). In general, *Kluyveromyces* species have been widely studied for fermentation, using glucose and lactose (from cheese whey) as carbon source (Walker and O'Neill, 1989). One species of *K. marxianus* (#3886 from a Portuguese Yeast Collection) has been reported to be salt-tolerant at concentrations of 2M (11.7%

w/v) NaCl compared with *S. cerevisiae* (Lages *et al*, 1999). On this specific research work, an extended study of 42 different species of yeast was made regarding osmotolerance under extreme saline conditions, but no study for yeast viability under those conditions was presented (Lages *et al*, 1999).

Figures from 28 to 31 show the effect of 4, 6, and 10% w/v NaCl on cell growth of 3 *Saccharomyces* industrial wine yeast strains and 2 non *Saccaharomyces* strains

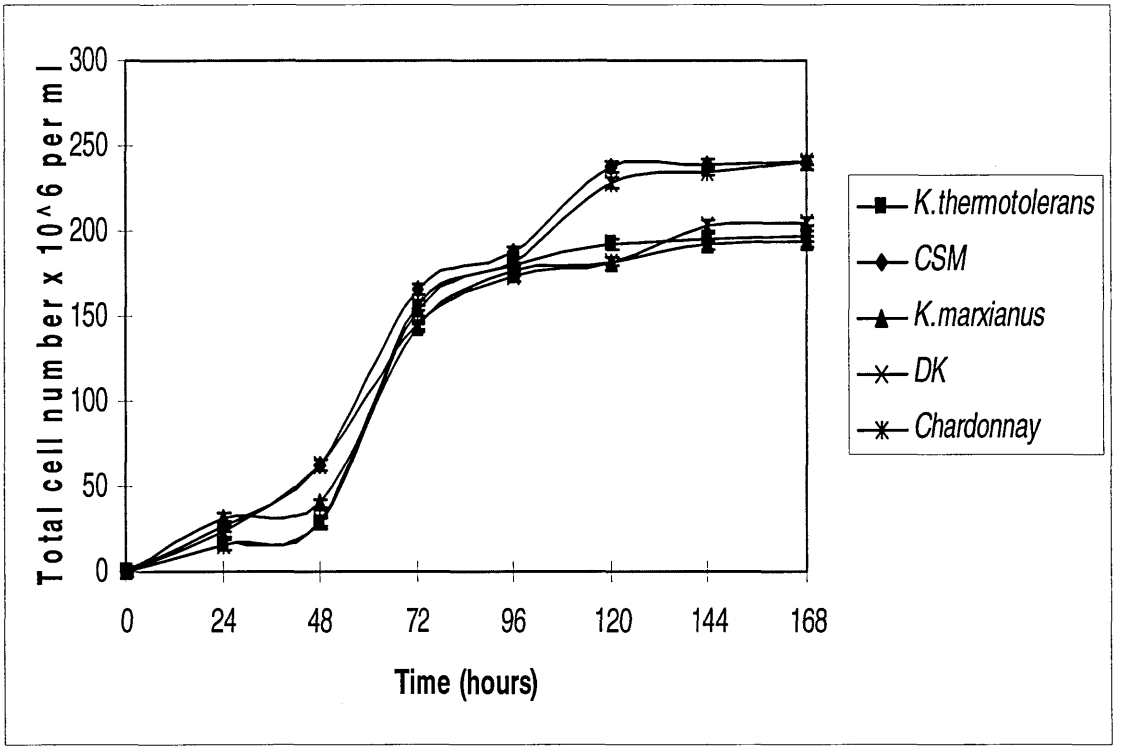


Figure 28 Cell growth for three *Saccharomyces* and two non-*Saccharomyces* strains
Three strains of *Saccharomyces cerevisiae* (strains Chardonnay, CSM and DK from Martin Vialate, France kindly provided by Ampeloiniki S.A. Thessaloniki Greece) and two non *Saccharomyces* (*K. thermotolerans* and *K. marxianus* (from the yeast culture collection of University of Abertay Dundee) were grown in glucose-based defined medium, without shaking at 25°C. Yeast cell growth was determined using haemocytometer (Thoma type) and yeast viability was assessed using the methylene blue method at the intervals shown. Standard error was from 2.6 to 3.6 %.

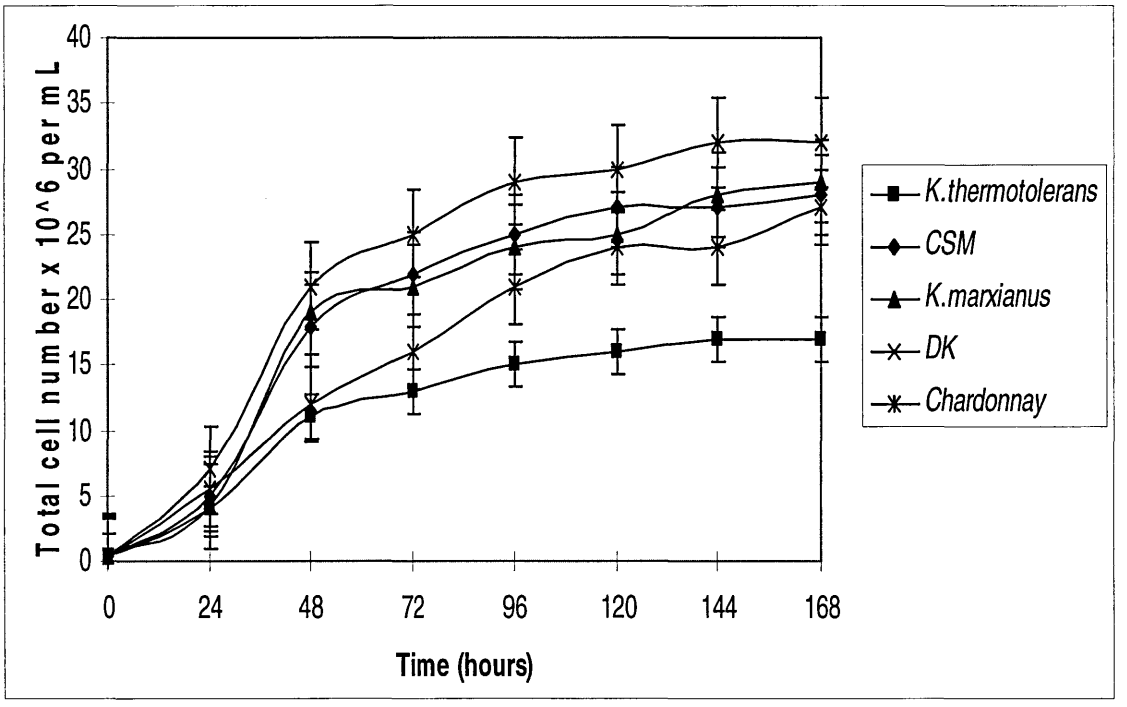


Figure 29 Cell growth for three *Saccharomyces* and two non-*Saccharomyces* strains treated with 4% w/v NaCl
Three strains of *Saccharomyces cerevisiae* (strains Chardonnay,CSM and DK from Martin Vialate, France kindly given by Ampeloiniki S.A. Thessaloniki Greece) and two non *Saccharomyces* (*K. thermotolerans* and *K. marxianus* (from the yeast culture collection of University of Abertay Dundee) were grown in glucose-based defined medium, without shaking at 25°C. Yeast cell growth was determined using haemocytometer (Thoma type) and yeast viability was assessed using the methylene blue method at the intervals shown. Standard error was from 1.75 to 3.3%.

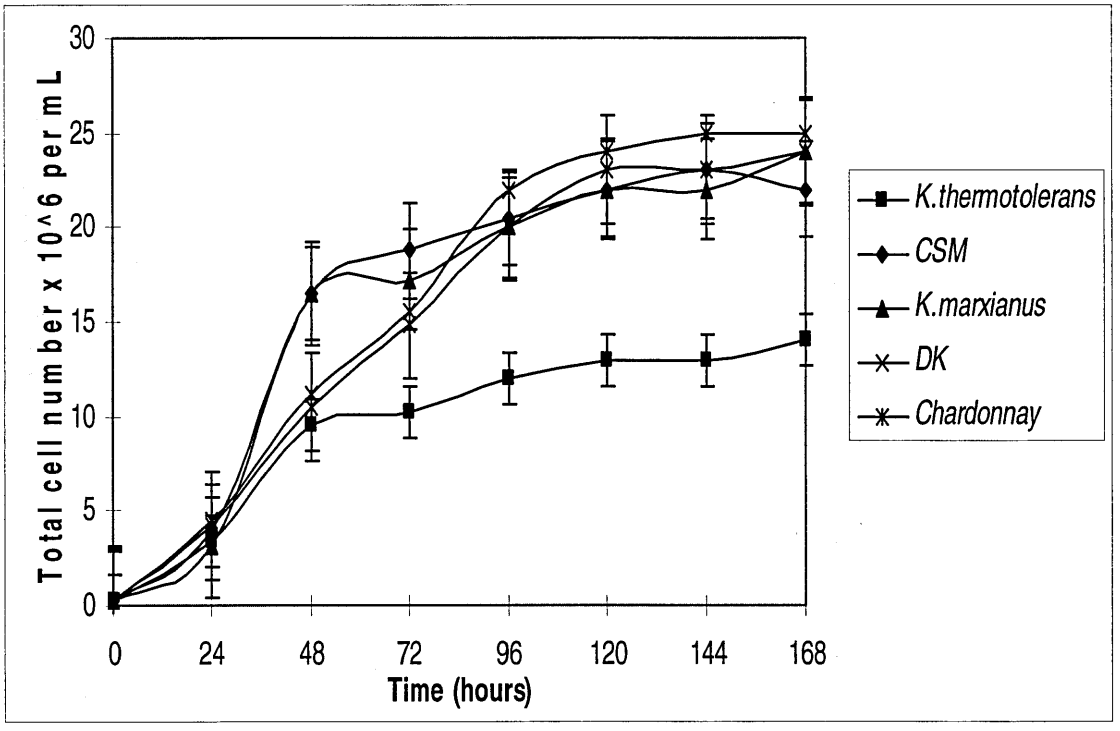


Figure 30 Cell growth for three *Saccharomyces* and two non-*Saccharomyces* strains under 6% of NaCl

Three strains of *Saccharomyces cerevisiae* (strains Chardonnay,CSM and DK from Martin Vialate ,France kindly given by Ampeloiniki S.A. Thessaloniki Greece) and two non *Saccharomyces* (*K. thermotolerans* and *K. marxianus* (from the yeast culture collection of University of Abertay Dundee) were grown in glucose-based defined medium, without shaking at 25°C. Yeast cell growth was determined using haemocytometer (Thoma type) and yeast viability was assessed using the methylene blue method at the intervals shown. Standard error was from 1.36 to 3.03%.

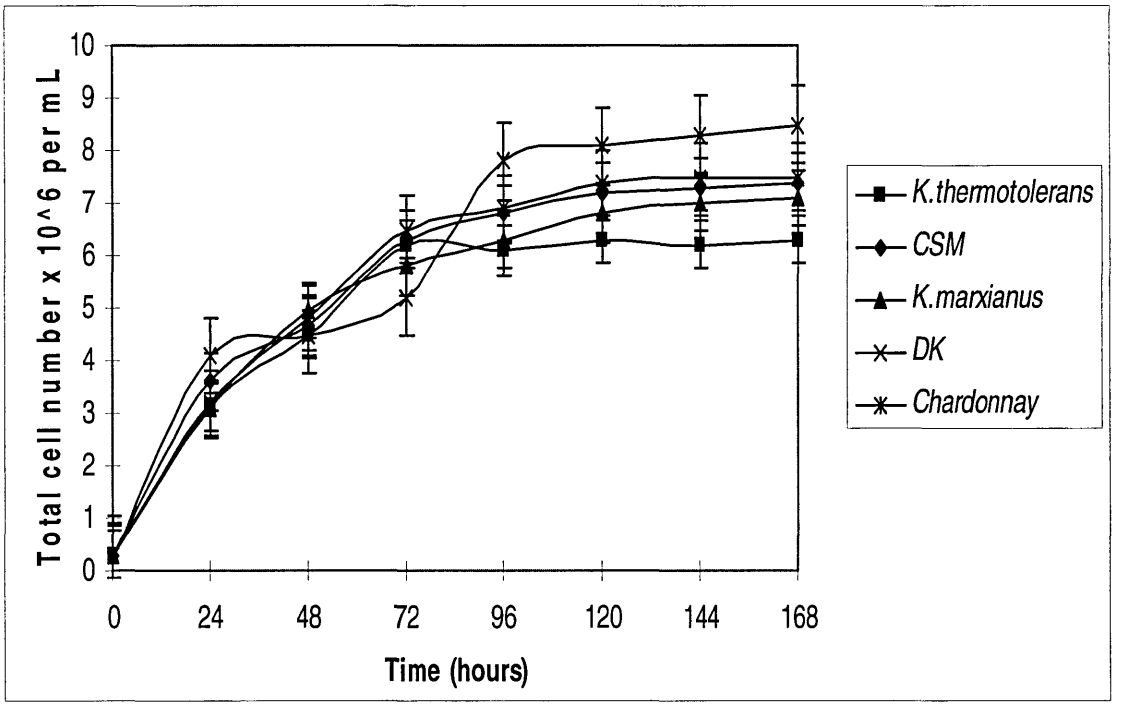


Figure 31 Cell growth for three *Saccharomyces* and two non-*Saccharomyces* strains under 10% of NaCl

Three strains of *Saccharomyces cerevisiae* (strains Chardonnay,CSM and DK from Martin Vialate, France kindly given by Ampeloiniki S.A. Thessaloniki Greece) and two non *Saccharomyces* (*K. thermotolerans* and *K. marxianus* (from the yeast culture collection of University of Abertay Dundee) were grown in glucose-based defined medium, without shaking at 25°C. Yeast cell growth was determined using haemocytometer (Thoma type) and yeast viability was assessed using the methylene blue method at the intervals shown.. Standard error was from 1.36 to 3.03%.

Figs 28 to 31 show that sodium chloride caused similar growth arrest phenomena for the five yeast strains studied. For example for *K. marxianus* the growth was almost the same as the *S.cerevisiae* strains. However, *K. thermotolerans* demonstrated a difference in growth especially at 4% and 6% w/v NaCl. For the highest concentration of 10% m/v of sodium chloride, the growth curve appears to very similar for all five species.

The phenomena of growth arrest caused by different sodium chloride concentrations, is represented in Figs 32-41. Each Fig represents the affect of osmotic stress on each yeast strain separately.

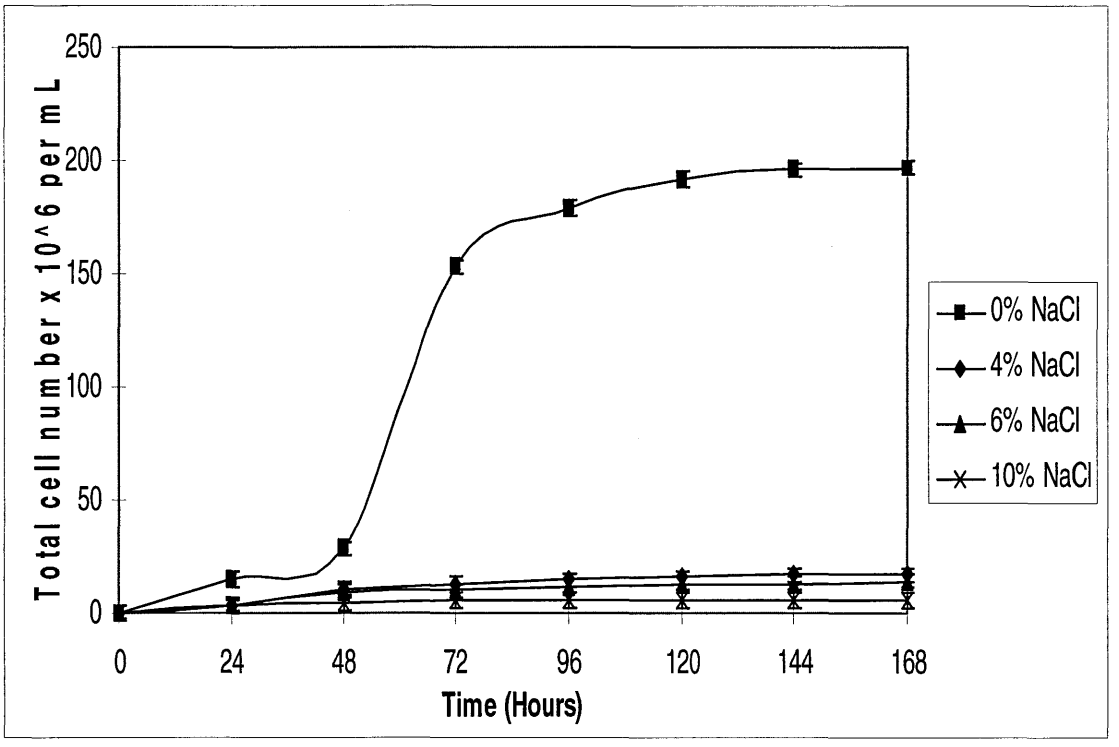


Figure 32 Cell growth of *Kluyveromyces thermotolerans* under osmotic stress conditions

K. thermotolerans (from the yeast culture collection of University of Abertay Dundee)was grown in glucose-based defined medium, without shaking at 25°C. Yeast cell growth was determined using haemocytometer (Thoma type) and yeast viability was assessed using the methylene blue method at the intervals shown.. Standard error was from 3.1 to 2.9%.

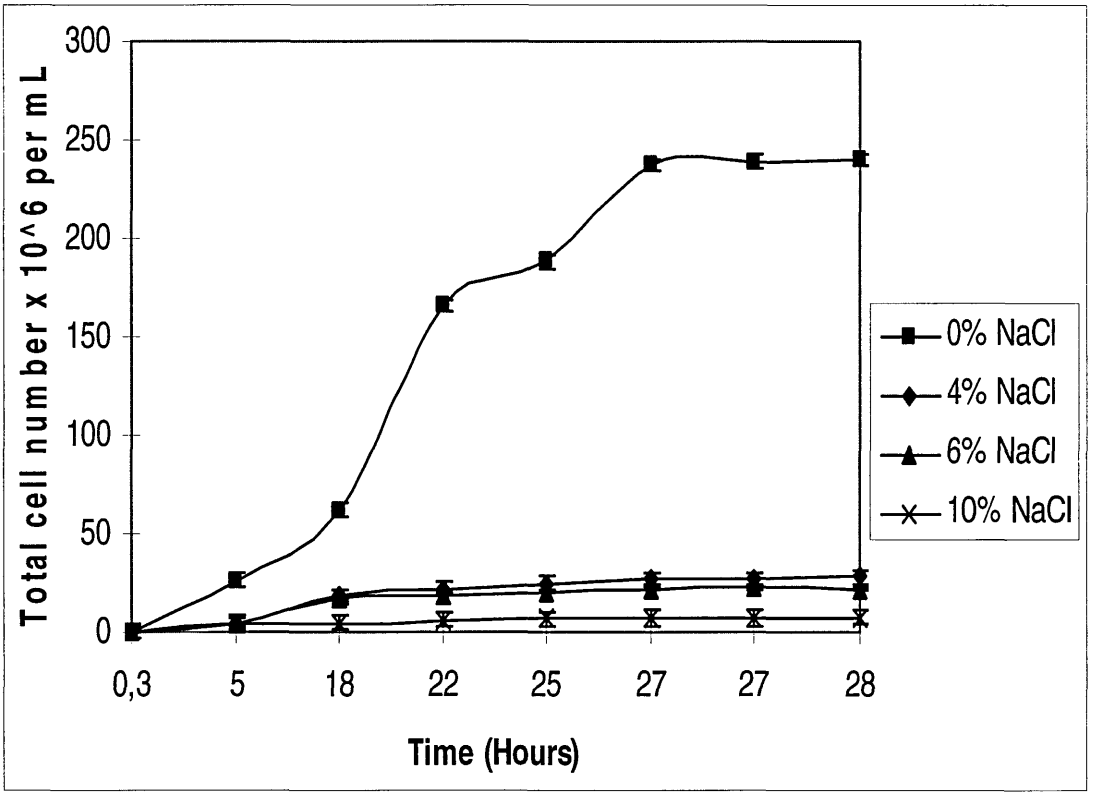


Figure 33 Cell growth of *S.cerevisiae CSM* under osmotic stress conditions

S. cerevisiae CSM (Martin Vialate , France) was grown in glucose-based defined medium, without shaking at 25°C. Yeast cell growth was determined using haemocytometer (Thoma type) and yeast viability was assessed using the methylene blue method at the intervals shown.. Standard error was from 0.88 to 3.78%.

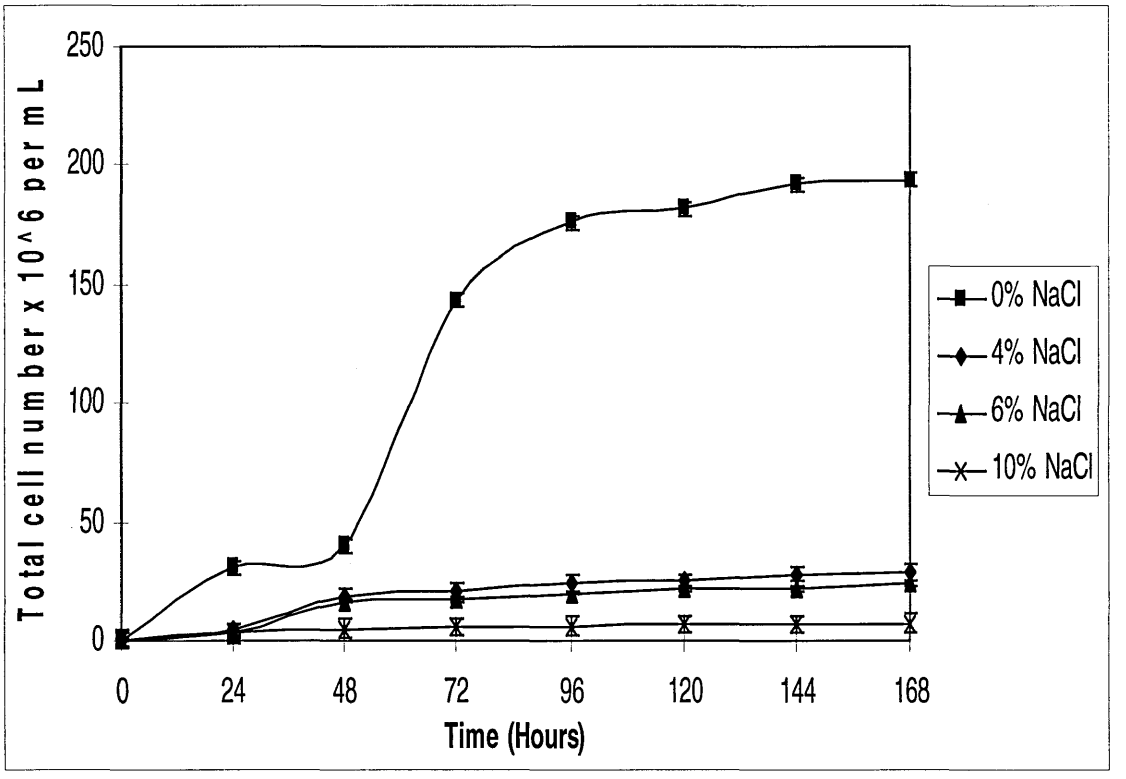


Figure 34 Cell growth of *Kluyveromyces marxianus* under osmotic stress conditions

K. marxianus (from the culture collection of University of Abertay Dundee)was grown in glucose-based defined medium, without shaking at 25°C. Yeast cell growth was determined using haemocytometer (Thoma type) and yeast viability was assessed using the methylene blue method at the intervals shown. Standard error was from 0.87 to 3.1%.

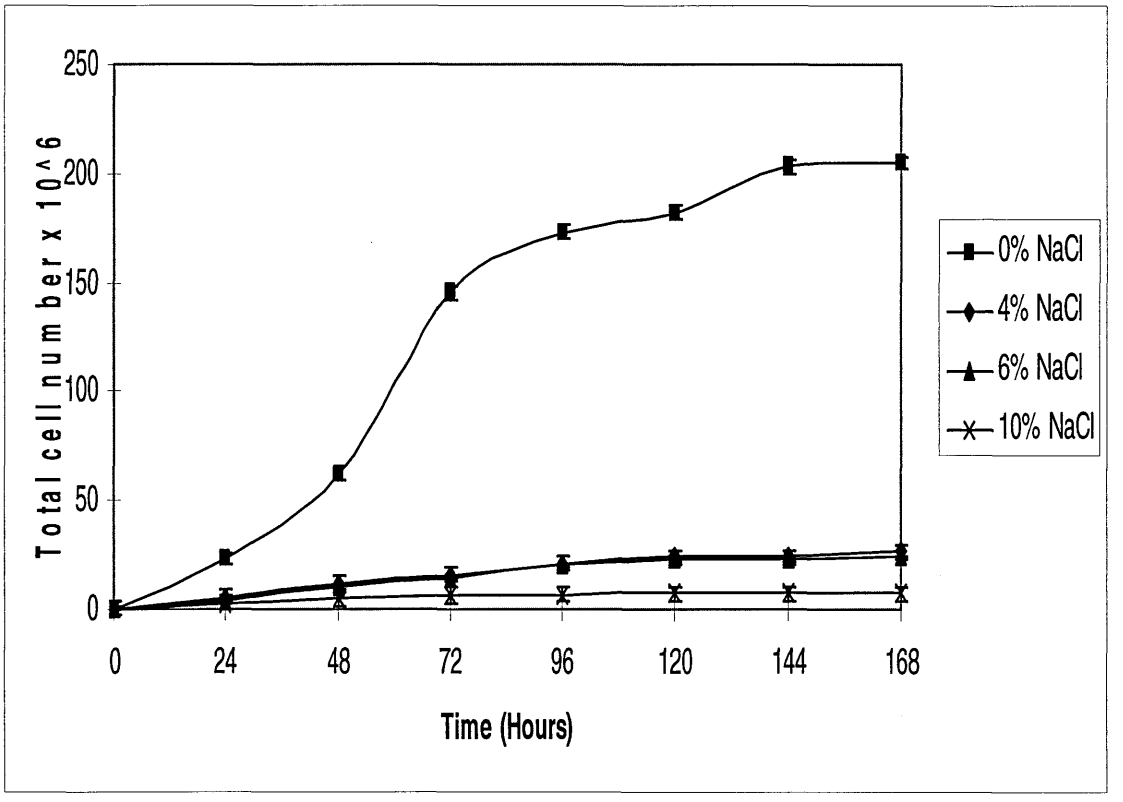


Figure 35 Cell growth of S. *cerevisiae DK* under osmotic stress conditions

S.cerevisiae DK (Martin Vialate , France)was grown in glucose-based defined medium, without shaking at 25°C. Yeast cell growth was determined using haemocytometer (Thoma type) and yeast viability was assessed using the methylene blue method at the intervals shown.. Standard error was from 0.93 to 3.32%.

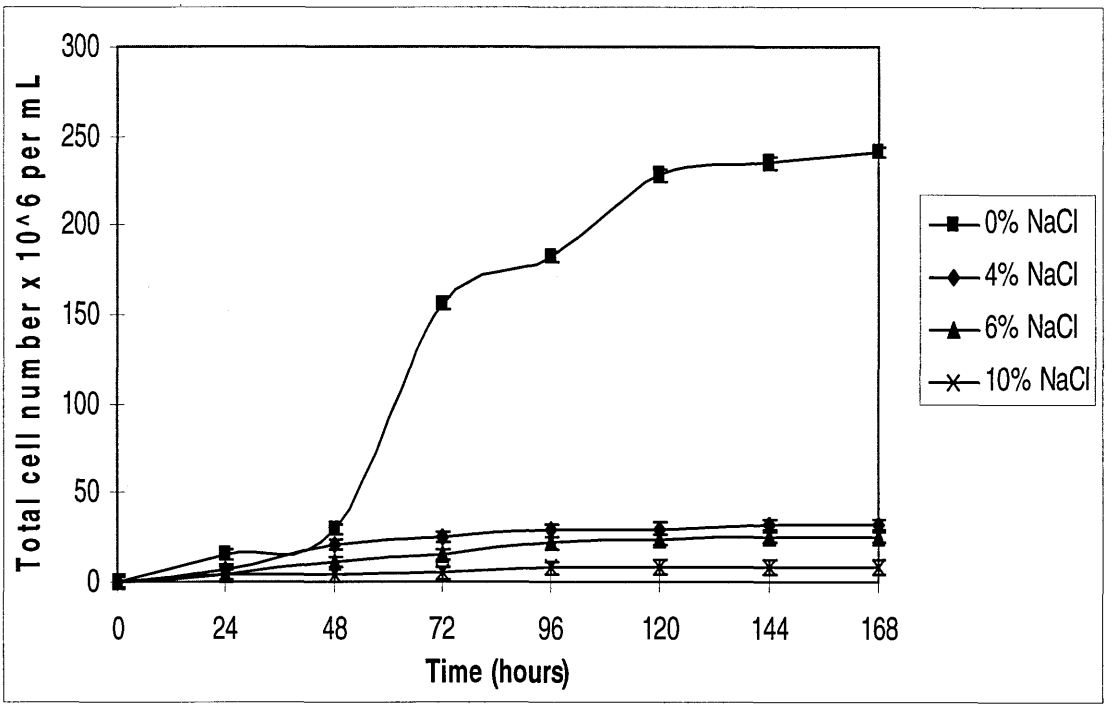


Figure 36 Cell growth of *S. cerevisiae Chardonnay* under osmotic stress conditions

S.cerevisiae Chardonnay (Martin Vialate, France) was grown in glucose-based defined medium, without shaking at 25°C Yeast cell growth was determined using haemocytometer (Thoma type) and yeast viability was assessed using the methylene blue method at the intervals shown.. Standard error was from 0.93 to 3.32%.

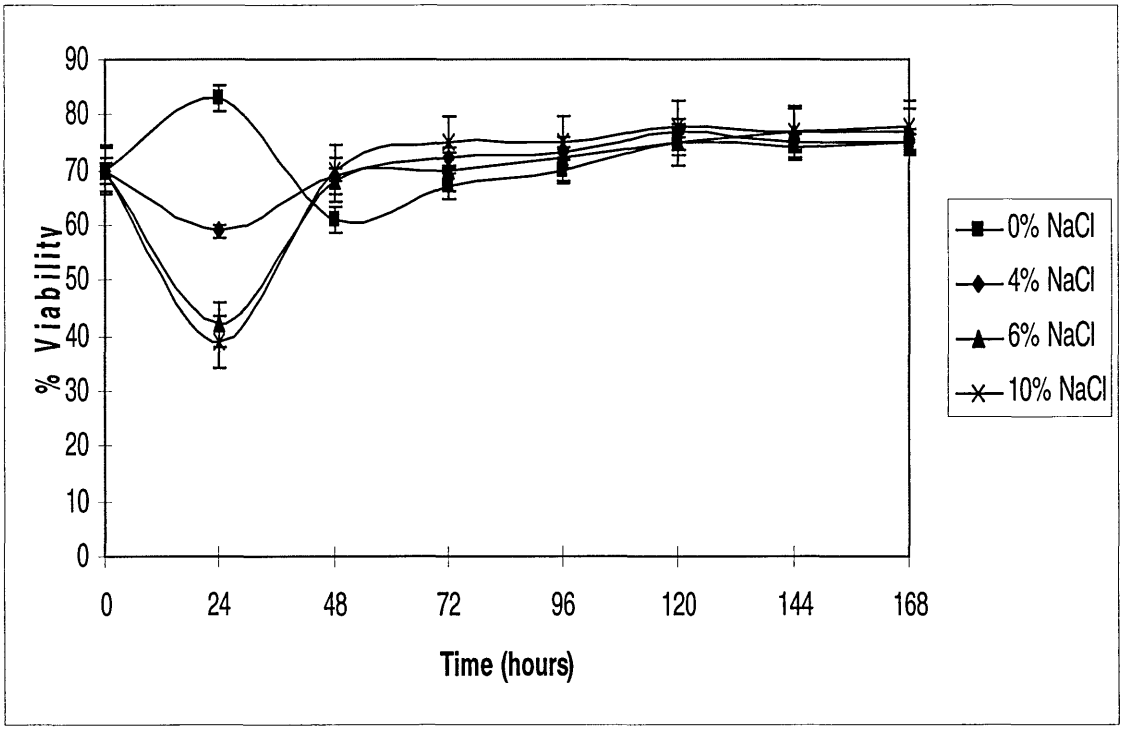


Figure 37 Influence of NaCl on yeast cell viability of *Kluyveromyces thermotolerans*

K. thermotolerans (from the yeast culture collection of University of Abertay Dundee)was grown in glucose-based defined medium, without shaking at 25°C. Yeast cell growth was determined using haemocytometer (Thoma type) and yeast viability was assessed using the methylene blue method at the intervals shown.. Standard error was from 1.98 to 4.69%.

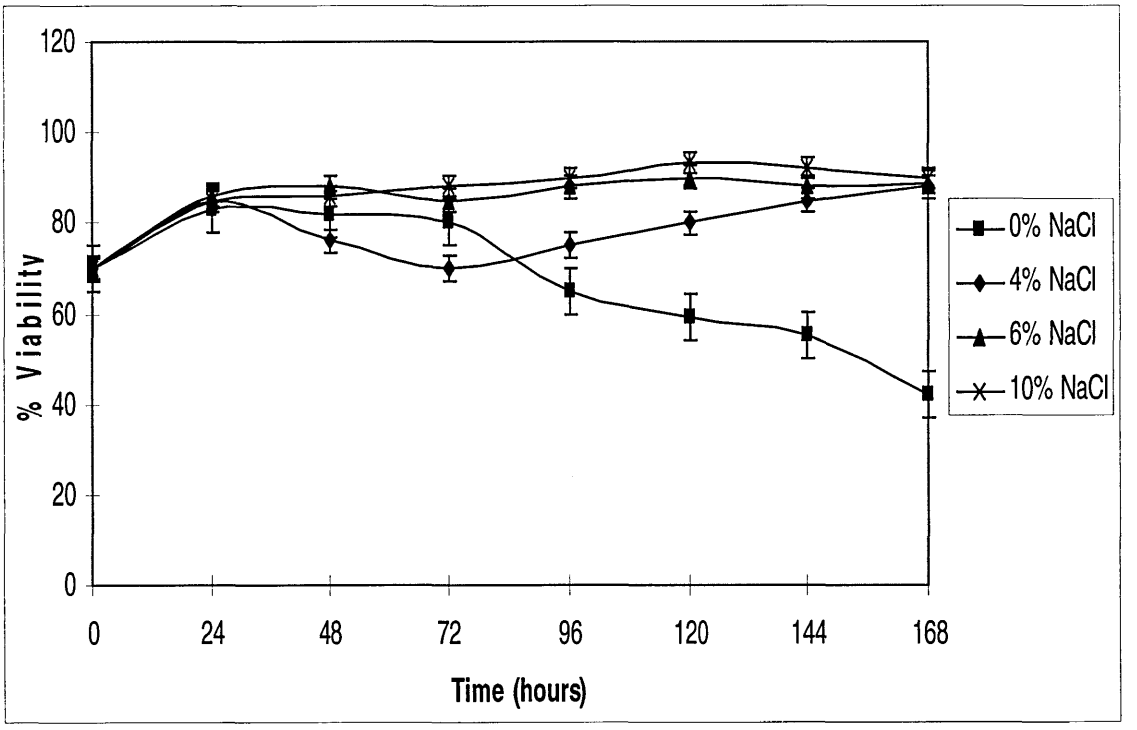


Figure 38 Influence of NaCl on yeast cell viability of *S.cerevisiae CSM*

S.cerevisiae CSM (Martin Vialate , France)was grown in glucose-based defined medium, without shaking at 25°C Yeast cell growth was determined using haemocytometer (Thoma type) and yeast viability was assessed using the methylene blue method at the intervals shown.. Standard error was from 2.28 to 5.16%.

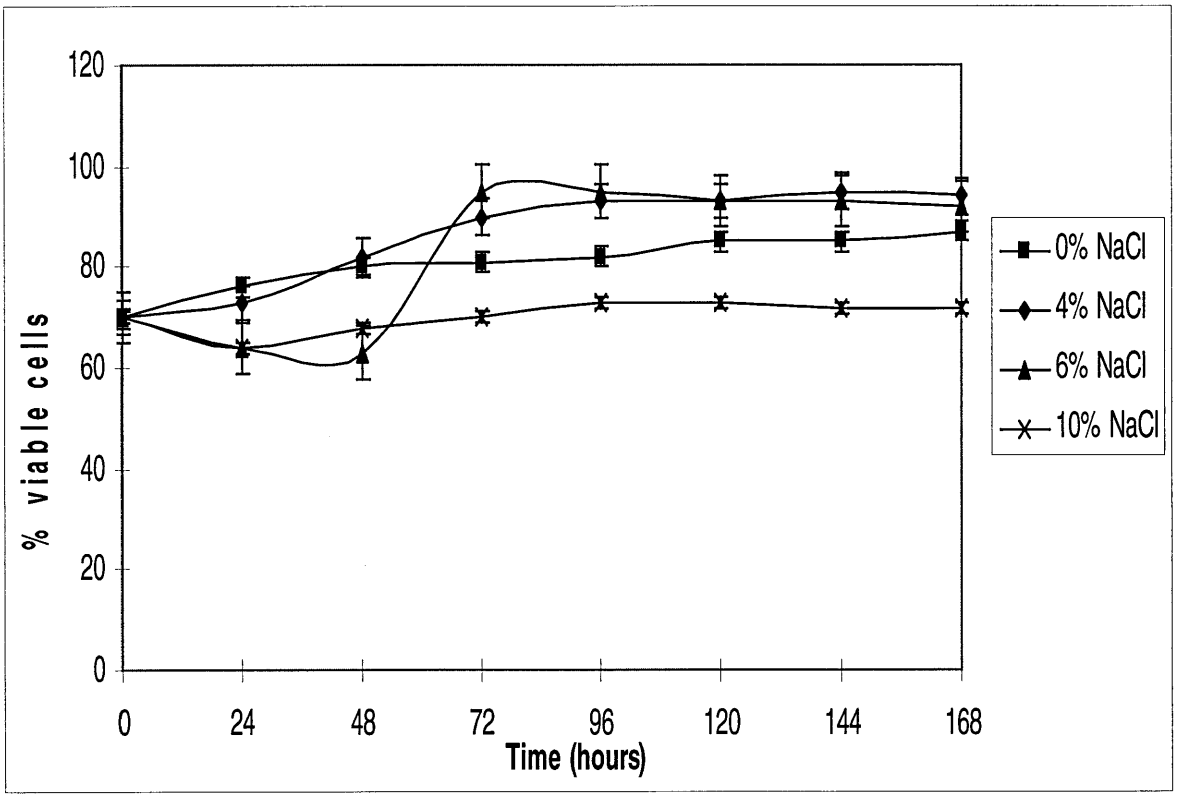


Figure 39 Influence of NaCl on yeast cell viability of *Kluyveromyces marxianus*

Kluyveromyces marxianus (from the yeast culture collection of University of Abertay Dundee) was grown in glucose-based defined medium, without shaking at 25°C. Yeast cell growth was determined using haemocytometer (Thoma type) and yeast viability was assessed using the methylene blue method at the intervals shown. Standard error was from 1.08 to 5.17%.

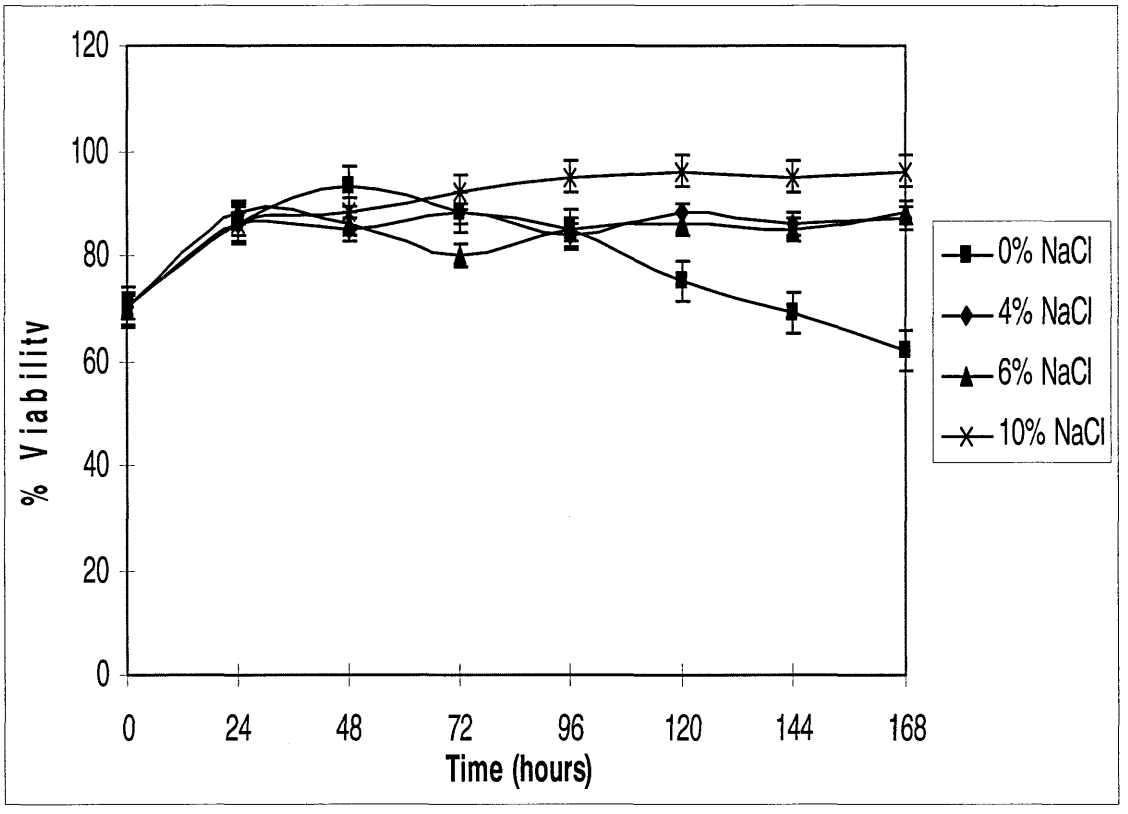


Figure 40 Influence of NaCl on yeast cell viability of *S.cerevisiae KD*
S.cerevisiae KD (Martin Vialate , France)was grown in glucose-based defined medium, without shaking at 25°C Yeast cell growth was determined using haemocytometer (Thoma type) and yeast viability was assessed using the methylene blue method at the intervals shown. Standard error was from 2.09 to 3.88%.

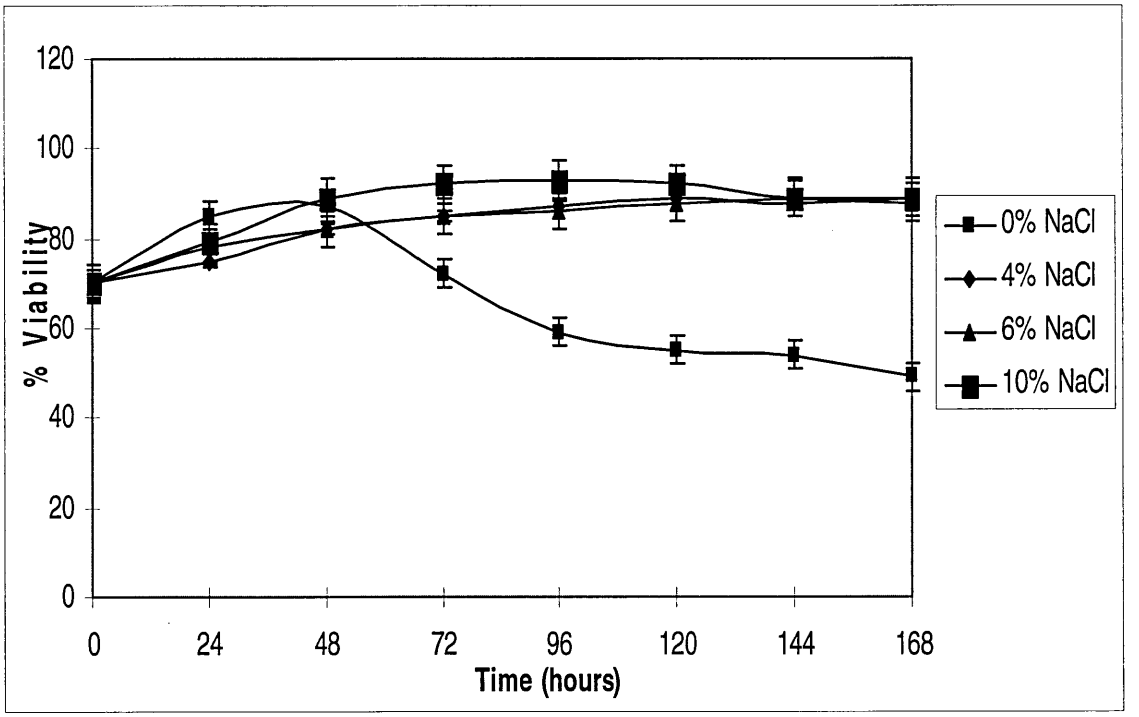


Figure 41 Influence of NaCl on yeast cell viability of *S.cerevisiae Chardonnay*

S.cerevisiae Chardonnay (Martin Vialate, France)was grown in glucose-based defined medium, without shaking at 25°C Yeast cell growth was determined using haemocytometer (Thoma type) and yeast viability was assessed using the methylene blue method at the intervals shown. Standard error was from 2.09 to 3.88%.

These results show that *K. thermotolerans* does not appear unduly affected by the high or low sodium chloride concentrations with regard to viability of the cells. At the beginning and at the end of fermentation cell viability was almost the same for the cells cultivated under osmotic stress conditions and without sodium chloride. The same phenomena also occurred for *K. marxianus* but for salt concentrations of 10% w/v it is apparent that viability was almost the same as that of the inoculum. For concentrations of 4% w/v and 6% w/v, cell viability was higher than the viability of the inoculum. For the two species of *Kluyveromyces* there appeared to be minimal effects of salt induced osmotic stress on yeast cell viability.

The three species of *Saccharomyces* demonstrated similar phenomena. Without NaCl treatment, cell viability decreased over time for all species. However, at all sodium chloride concentrations, an increased cell viability was demonstrated, even at the highest sodium chloride concentration of 10% w/v. Especially for *S.cerevisiae Chardonnay* and for *S.cerevisiae CSM* strains the viability at the end of the fermentation was the same for all NaCl concentrations and close to 90%. For *S.cerevisiae DK* the highest viability occurred at 10% NaCl treatments. Overall, these results show that salt pre-conditioning of yeast, especially *S.cerevisiae* species, had a positive affect on viability of the cells.

3.3 Fermentation performance of NaCl pre-conditioning yeast cells in osmotic stress conditions imposed by different glucose concentrations

The main hypothesis in this section is that a mild salt-stress has a beneficial effect on yeast physiology and "prepares" the cells for other more stressful conditions, particularly those occurring in high sugar fermentations.

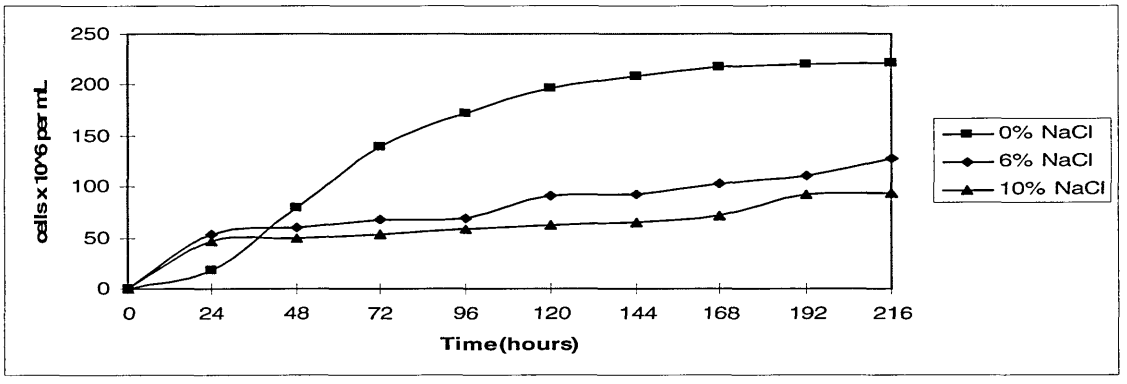
It can be hypothesized that treatment of the yeast with 6% and 10% NaCl may have a positive effect on sugar-related osmotolerance of yeast cells. We therefore performed fermentations by progressively increasing the media glucose concentrations. These experiments aimed to observe the capability of salt pre-conditioned cells to grow and ferment high sugar concentrations in comparison to non-salt pre-conditioned yeast cells. After 400h fermentation, media containing 20% w/v D-glucose was inoculated with $5x10^6$ living cells per mL pre-conditioned in 6% and 10% w/v NaCl. Subsequently, media containing 30, then 40% w/v D-glucose were inoculated in a similar manner. Yeast cell growth and viability were monitored.

In the following section and in Figures 42 to 44 we demonstrate the ability of salt preconditioned yeast cultures to ferment gradually increased sugar concentration (200 g/L to 400 g/L).

The following figures show the growth of preconditioned yeast cells and untreated cells during fermentations. For the untreated cells the growth decreased from the first fermentation to the second and then to the third (see section 2.1.4). Actually the total cell number at the third fermentation is less than that of the cells which had treated under

osmotic stress conditions before the first inoculation.

A



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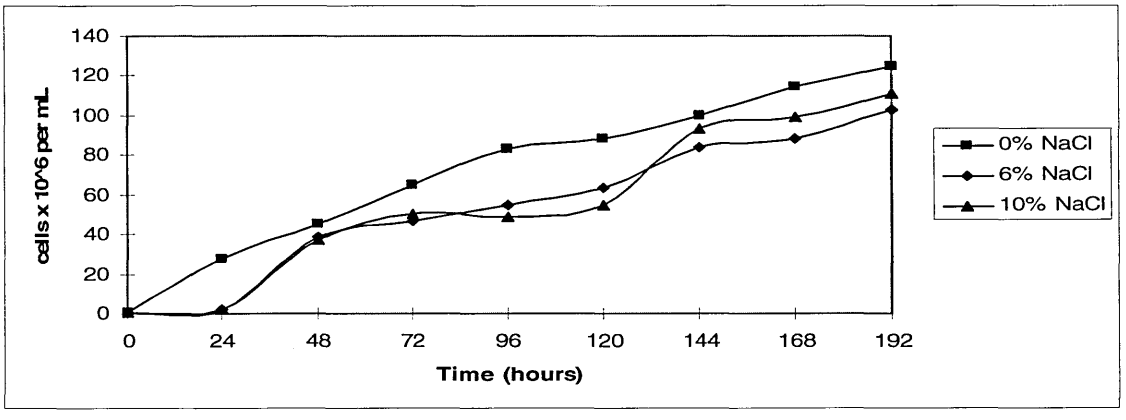

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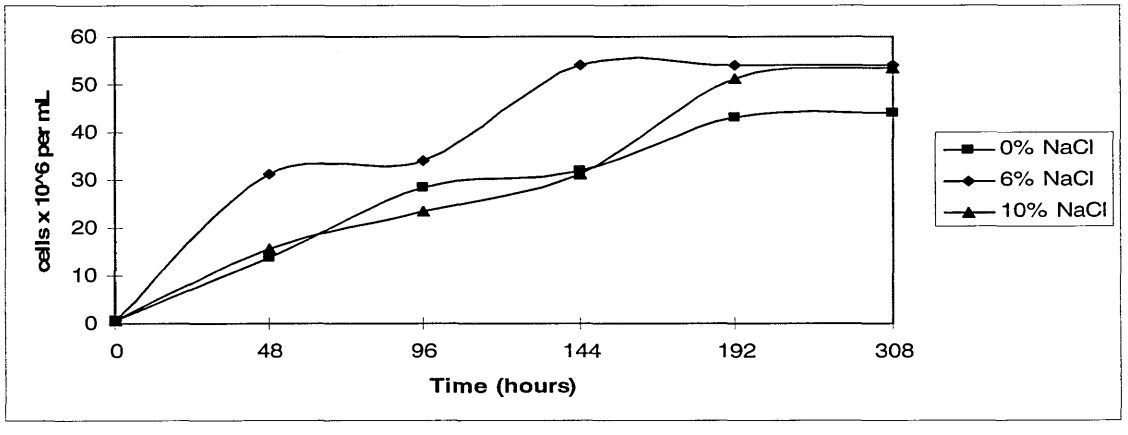
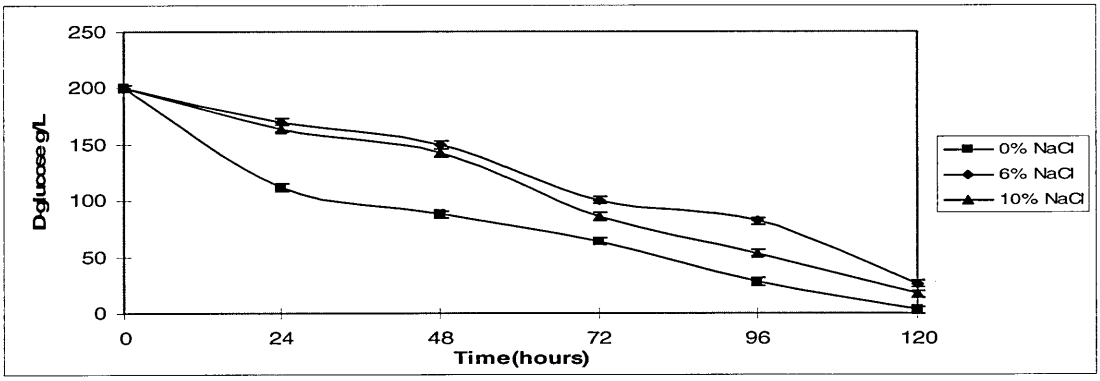


Figure 42 Yeast growth under increased glucose concentration following salt-preconditioning.
Cells of *Vin 13* were preconditioned in salt (0,6,10% w/v NaCl) grown in glucose-based defined medium containing an increased concentration (200,300,400 g/L) of D-glucose, without shaking at 25°C. Yeast cell growth was determined using a haemocytometer at the intervals shown. Standard error was from 2.09 to 3.88.

Were **A** is for 200 g/L of D-glucose, **B** for 300 g/L of D-glucose and **C** for 400 g/L of D-glucose.
Figure 42 shows that for 40% w/v D-glucose, the fermentation time was 116 hours longer and the total cell number was almost half compared with the other two fermentations of 20 and 30% w/v D-glucose. Growth in media with 20% and 30% D-glucose was similar. The most important observation was that for higher glucose concentrations (30% and 40%) where the total cell number was observed to be close to $5x10^7$/ml for the fermentation of 40% D-glucose and close to 10 $x10^7$for the fermentation of 30% D-glucose.

These results were surprising because at these high sugar concentrations, the osmotic influence of sugar can delay the onset of fermentation. The resulting partial plasmolysis of yeast cells may be one of the causes of a lag period prior to active fermentation (Nishino *et al.*, 1985).
These previously published findings do not conform with present results regarding the high growth of the yeast cells in glucose osmostress conditions. It is therefore proposed that salt pre-conditioning was induce the biosynthesis of compatible solutes (see section 1.3.2 and 1.3.3) to protect the cells under high sugar concentrations. This has implications for fermentation industries and is further explored in section 3.3 regarding practical winemaking.

A



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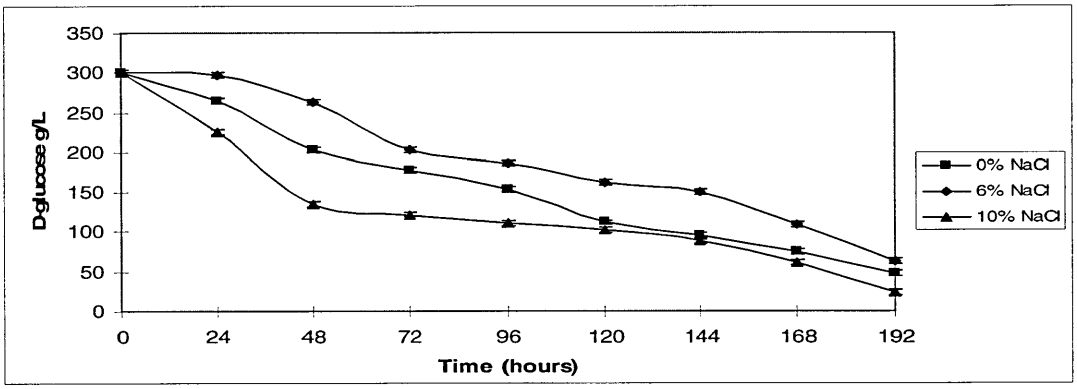

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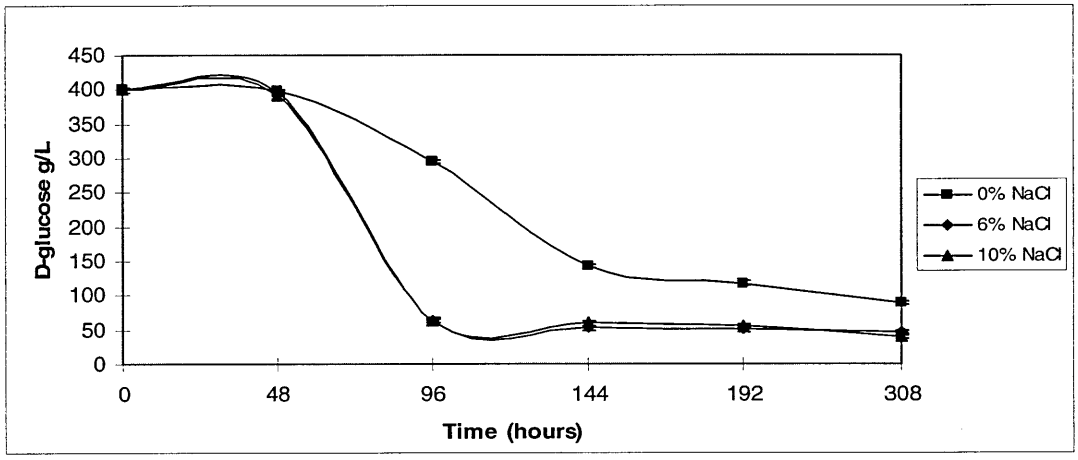


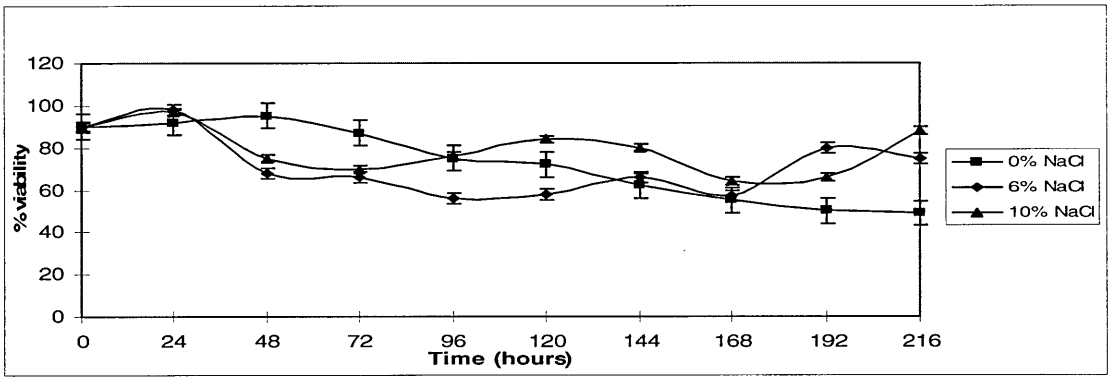
Figure 43 Sugar consumption under increased glucose concentration following salt-preconditioning of yeast

Cells of *Vin 13* were preconditioned in salt (0,6,10% w/v NaCl) grown in glucose-based defined medium containing an increased concentration (200,300,400 g/L) of D-glucose, without shaking at 25°C. Sugar consumption was determined by the DNS method and the standard error was between 1.09 and 3.03%. **A** represnts 200 g/L D-glucose, **B** 300 g/L D-glucose and **C** 400 g/L D-glucose.

Figure 43 shows that 20% D-glucose consumption was almost linear and the levels of residual sugars were the approximately the same, irrespective of yeast culture treatment. For the fermentation in media with 30% D-glucose, sugar consumption increased markedly when the inoculum cells were pre-conditioned with 10% w/v NaCl. At very high glucose levels (40% w/v), a rapid decrease in sugar consumption was observed between 48 and 96 hours, declining from 395-64g/L glucose and then remaining constant. This indicates that cells which have been pre-conditioned at levels lower than 6% and 10% w/v of NaCl displayed glucose utilisation and faster fermentation ability than cells which lacked any salt preconditioning. This phenomenon may be due to preconditioned cells inducing expression of certain stress-responsive genes (section 1.4.2). "Reactivation" of key genes encoding stress metabolites may act to protect cells under the osmotic stress conditions imposed by high sugar levels at the onset of fermentation.

Figure 44 describes the viability of yeast cells under repeat fermentation (see section 2.1.4). It can be seen that during the first fermentation with 200g/L glucose that the preconditioned cells retained a higher viability that the untreated cells. For the two other fermentations (300 and 400g/L glucose) at the end of fermentation the viability was almost the same as the control culture, and at low levels. Nevertheless, in both of these fermentations, at 24 hours a big difference regarding cell viability was observed between salt-preconditioned and untreated yeast cells, again reflecting a fairly rapid adaptation by the salt treated cells to the stressful environment.

A



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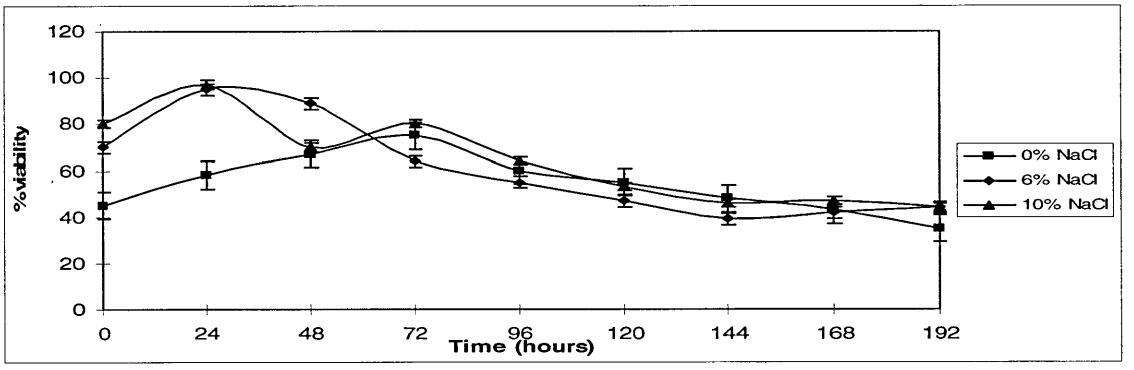

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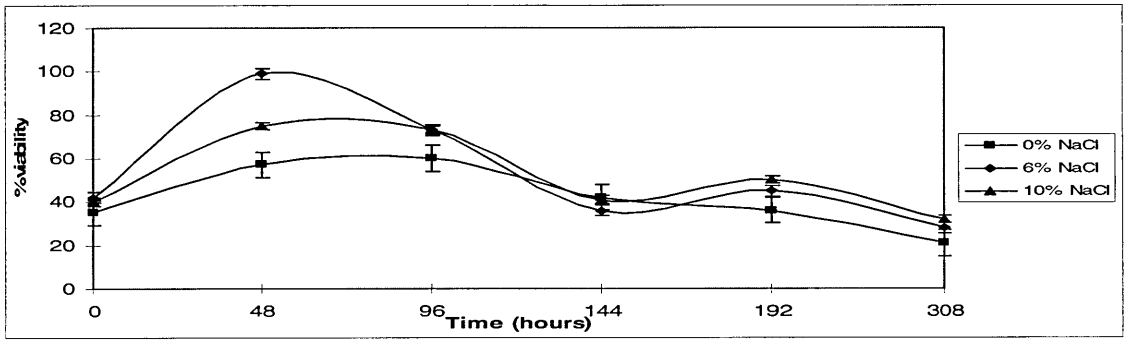


Figure 44 Yeast viability under increased glucose concentration following salt-preconditioning

Cells of *S. cerevisiae* (Vin 13) were preconditioned in salt (0,6,10% w/v NaCl) then grown in glucose-based defined medium containing an increased concentration (200, 300, 400 g/L) of D-glucose, without shaking at 25°C. Yeast cell growth was determined using haemocytometer (Thoma type) and yeast viability was assessed using the methylene blue method at the intervals shown. Standard error was from 1.75 to 5.8%.

A represents 200 g/L D-glucose, **B** 300 g/L D-glucose and **C** 400 g/L D-glucose.

These results show that in general yeast cell viability decreased when the sugar levels increased. For 20% D-glucose fermentations, although yeast viability remained high even towards the end of the fermentation, a difference (of approx 20%) between preconditioned and unconditioned cells occurred. However, in 30% and 40% D-glucose fermentations, yeast viability remained low and all cultures demonstrated the same behaviour regarding viability. Only during the first 24 hours of fermentation differences (approx 20%) were observed (Fig 43b and c). Note that the yeast viability at the start of the very high glucose concentration (400 g/L) fermentation was low (around 40%), but this is due to the sequential nature of the experiment (i.e. cells transferred from low to high sugar media). From these results, it would appear that preconditioning of the cells under salt-induced osmotic stress conditions did not affect in a positive way the viability of the cells under those specific conditions. However, if we compare the growth ability and sugar utilization characteristics between preconditioned and unconditioned cells it can be concluded that salt-preconditioning led to enhanced fermentation capabilities in high sugar media (Figures 42 and 43). These findings have direct implications for industrial fermentations that employ feedstocks with elevated carbohydrate levels (eg. Sauterne-type and Botrytised type wines, high-gravity brewing, bioethanol from molasses and starch hydrolysates). The following section focuses on industrial-scale fermentations in relation to winemaking, and specifically the influence of salt-preconditioned wine yeasts on fermentation performance.

3.4 Industrial scale fermentations

During this part of the research, experiments were conducted on an industrial scale using the same four industrial yeast strains as per lab-based fermentations. Strain *S. cerevisiae* VIN 13 was employed for the 2005 winemaking season using must from Chardonnay variety grapes; strain *S. cerevisiae* Vitilevure Chardonnay was used for winemaking years 2006, 2007 and 2008. Additionally strains *S. cerevisiae* Vitilevure KD and *S. cerevisiae* Vitilevure CSM were employed for the fermentation of Merlot grape must and for re-inoculation of stuck fermentations for a tank of Syrah grape must. For the years 2005, 2006 and 2007 we used NaCl pre-conditioned and unconditioned yeast cells for inoculation of 5000L stainless steel tanks and for the year 2008 we used only pre-conditioned yeast cells. Pre-conditioning of the cells was conducted by treating cells with 6% w/v NaCl for 16 hours in the inoculum volume (200L). This concentration of sodium chloride was chosen following promising data from the lab scale experiments (see Figs 13 and 14) that showed better yeast viability and maximum growth compared with higher salt conditions.

For all winemaking years, results were compared with the "standard" given by the yeast-producing companies (Anchor and Martin Vialate) for each yeast strain. We aimed to show that pre-conditioning under salt stress conditions for cells had a positive affect regarding: alcohol productivity, fermentation yield (regarding sugar consumption) and sugar and temperature stress tolerance. The latter conditions involved

cells withstanding fermentation temperatures for every year of 9-10 °C and sugar concentrations higher than 300 g/L.

2005 Industrial fermentations

Figures 45-47 demonstrate the fermentative behaviour of the VIN 13 yeast strain regarding sugar consumption, alcohol production and glycerol production The results compare the behaviour of the same strain with cells which were pre-conditioned under salt stress conditions of 6% NaCl w/v for 16 hours before inoculation and those that were not pre-conditioned. From Table 3 and Figure 45 the pattern of sugar consumption for both cases was the same but regarding the residual sugars at the end of the fermentations the difference was around 30 g/L of D-glucose. Additionally, alcohol productivity and glycerol production were seen to have pronounced differences as well. For example, the alcohol content at the end of the fermentations differed by 3% v/v and the glycerol concentration by 10 g/L. These results show that salt-preconditioned yeast cells are able to ferment high sugar concentrations with low residual sugars at the end of the fermentation and can produce higher amounts of alcohol in comparison with the un-preconditioned cells. The higher levels of glycerol produced following preconditioning is significant in winemaking, especially for white wines since glycerol is related with the textural (mouthfeel, body) characteristics of such wines.

Table 3 Fermentation performance of salt pre-conditioned (6% NaCl w/v) wine yeast (VIN 13) in 2005 Chardonnay grape must. Fermentation performance (in 5000L vessels) was assessed by sugar consumption, alcohol and glycerol production. PCC represents pre-conditioned cells and UCC represents unconditioned cells.

Time (Hours)	Sugars g/L		Alcohol % v/v		Glycerol g/L	
	PCC	UCC	PCC	UCC	PCC	UCC
0	327	327	0	0	0	0
24	226	279	5.71	2.25	2.23	1.2
48	203	236	6.8	4.8	6.6	2.2
72	161	206	9.45	6.8	3.97	2.35
96	154	191	10.05	7.8	3.53	2.8
120	125	164	11.44	9.2	10.15	3.8
144	106	124	12.4	11.3	6.62	3.2
192	26	86	18.47	13.5	4.41	4.8
216	18	76	18.24	13.8	18.2	5.2
264	12	62	18.43	14.5	13.6	8.9
312	10	58	18.43	14.8	8.2	7.2
360	6	48	18.39	15.1	10.6	5.9
408	5	43	18.43	15.15	18.3	6.8
456	2,3	33	18.3	15.2	18.2	7.6

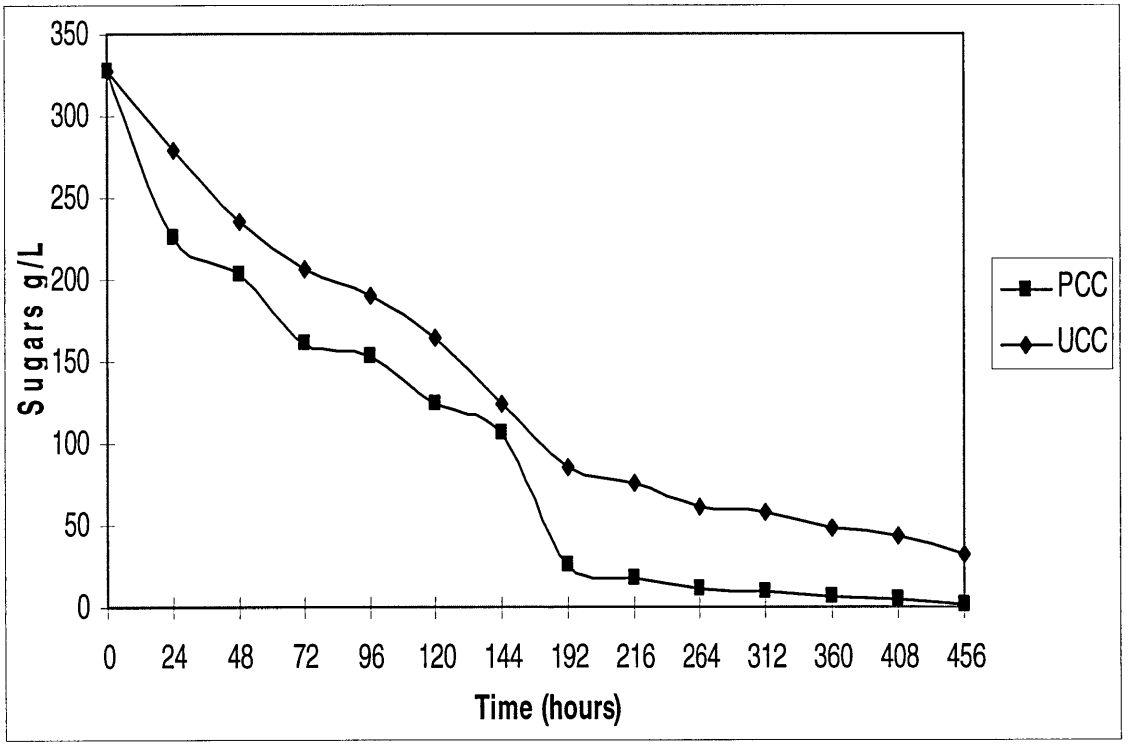


Figure 45 Residual sugars for salt pre-conditioned yeast during 2005 Chardonnay grape must fermentation
Fermentations were conducted with preconditioned yeast (strain VIN 13) using 6% NaCl and growing cells for 16 hours prior to inoculation of 5000 L stainless steel tanks containing Chardonnay must. Must was clarified by pectinolytic enzymes and filtered before inoculation. Fermentation by unconditioned yeast cells is also demonstrated.

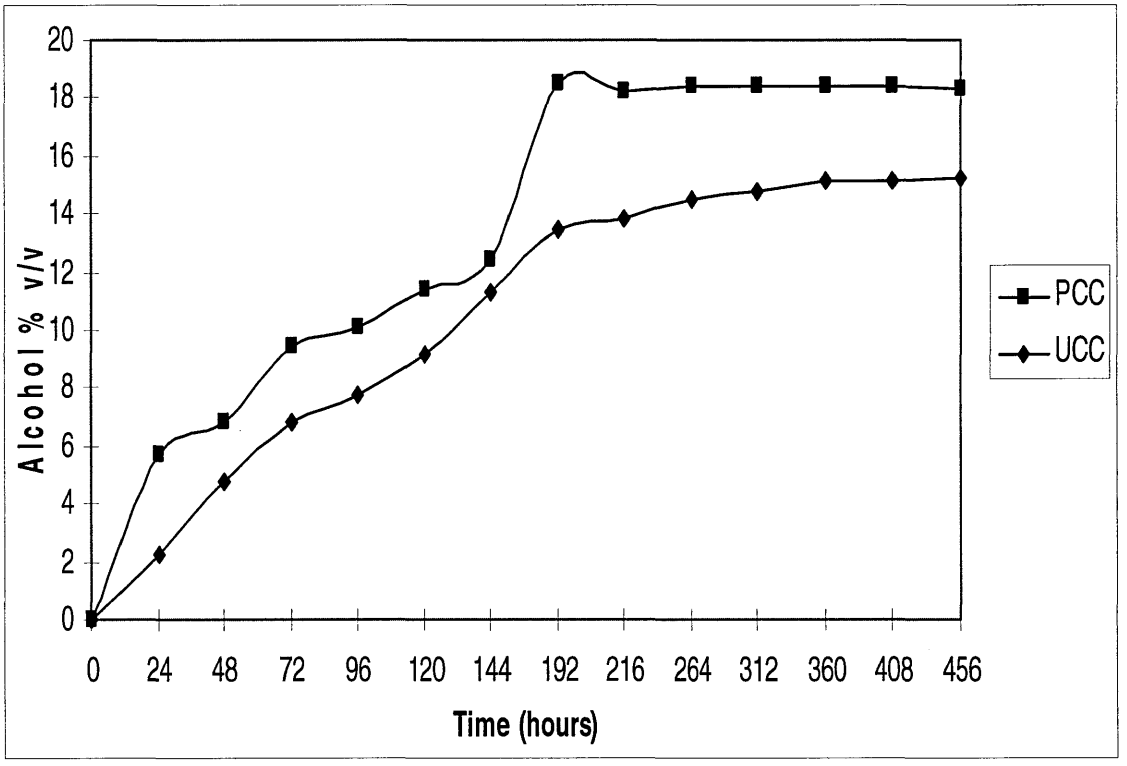


Figure 46 Alcohol production by salt pre-conditioned yeast during 2005 Chardonnay grape must fermentation
Fermentations were conducted with preconditioned yeast (strain VIN 13) using 6% NaCl and growing cells for 16 hours prior to inoculation of 5000 L stainless steel tanks containing Chardonnay must. Must was clarified by pectinolytic enzymes and filtered before inoculation. Fermentation by unconditioned yeast cells is also demonstrated.

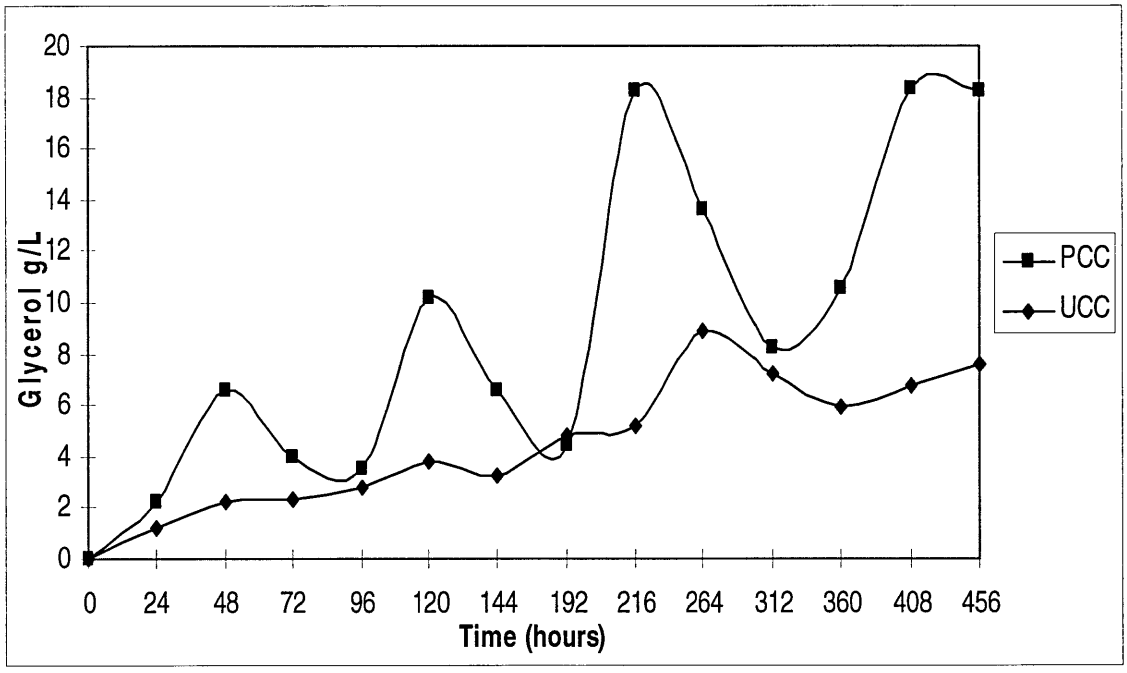


Figure 47 Glycerol production by salt pre-conditioned yeast during 2005 Chardonnay grape must fermentation
Fermentations were conducted with preconditioned yeast (strain VIN 13) using 6% NaCl and growing cells for 16 hours prior to inoculation of 5000 L stainless steel tanks containing Chardonnay must. Must was clarified by pectinolytic enzymes and filtered before inoculation. Fermentation by unconditioned yeast cells is also demonstrated.

2006 Industrial fermentations

Figures 48-50 demonstrate the fermentative behaviour of salt-preconditioned *S. cerevisiae* Vitilevure Chardonnay yeast strain regarding sugar consumption, alcohol production and glycerol production. The results compare the behaviour of the same strain cells which were pre-conditioned under salt stress conditions of 6% NaCl w/v for 16 hours before inoculation with those that were not pre-conditioned. From Table 4 and Figure 48 the pattern of sugar consumption for both cases was the same but regarding the residual sugars at the end of the fermentations the difference was about 25 g/L D-glucose. Additionally, results show that alcohol productivity and glycerol production displayed pronounced differences as well. For example, the alcohol content at the end of the fermentations differed by 1.5% v/v and the glycerol concentration by 9 g/L (Figure 50).

Table 4 Fermentation performance of salt pre-conditioned (6% NaCl w/v) wine yeast (Vitilevure Chardonnay) in 2006 Chardonnay grape must. Fermentation performance (in 5000L vessels) was assessed by sugar consumption, alcohol and glycerol production. PCC represents pre-conditioned cells and UCC represents unconditioned cells.

Time (Hours)	Sugars g/L		Alcohol % v/v		Glycerol g/L	
	PCC	UCC	PCC	UCC	PCC	UCC
0	314	314	0	0	0	0
24	198	259	6.8	2.6	1.95	1.38
48	164	239	8.2	3.9	5.8	2.8
72	138	226	9.8	4.8	2.97	4.5
96	132	208	10	5.9	3.9	3.9
120	97	194	11.2	6.7	8.9	4.6
144	69	176	12.25	7.8	5.8	5.2
168	46	152	13.1	8.6	6.9	4.2
192	19	136	16.8	9.8	4.2	3.6
216	14	106	17.25	11.8	16.8	5.8
264	13	82	17.3	12.9	12.9	5.4
312	11	71	17.6	13.6	14.5	6.2
360	8	38	17.55	15.8	13.8	6.9
408	6	32	17.5	15.85	16.9	7.3
456	2,4	27	17.4	15.9	17.2	8.8

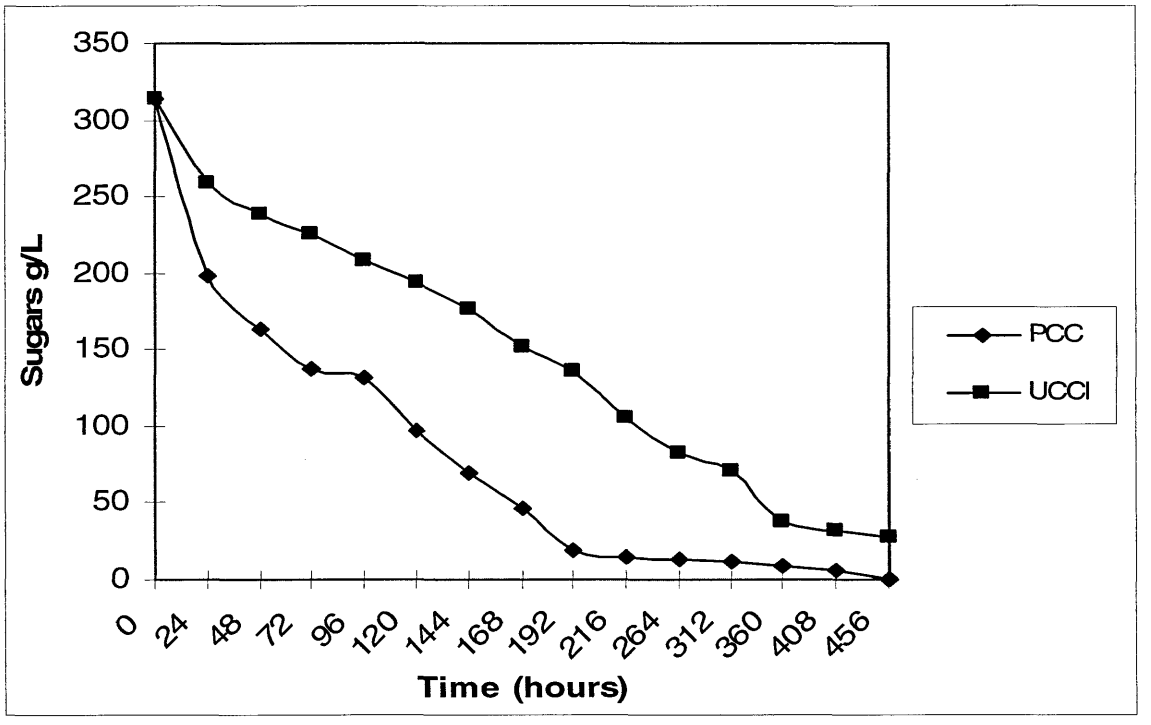


Figure 48 Residual sugars by salt pre-conditioned yeast during 2006 Chardonnay grape must fermentation
Fermentations were conducted with preconditioned yeast (Vitilevure Chardonnay) using 6% NaCl and growing cells for 16 hours prior to inoculation of 5000 L stainless steel tanks containing Chardonnay must. Must was clarified by pectinolytic enzymes and filtered before inoculation. Fermentation by unconditioned yeast cells is also demonstrated.

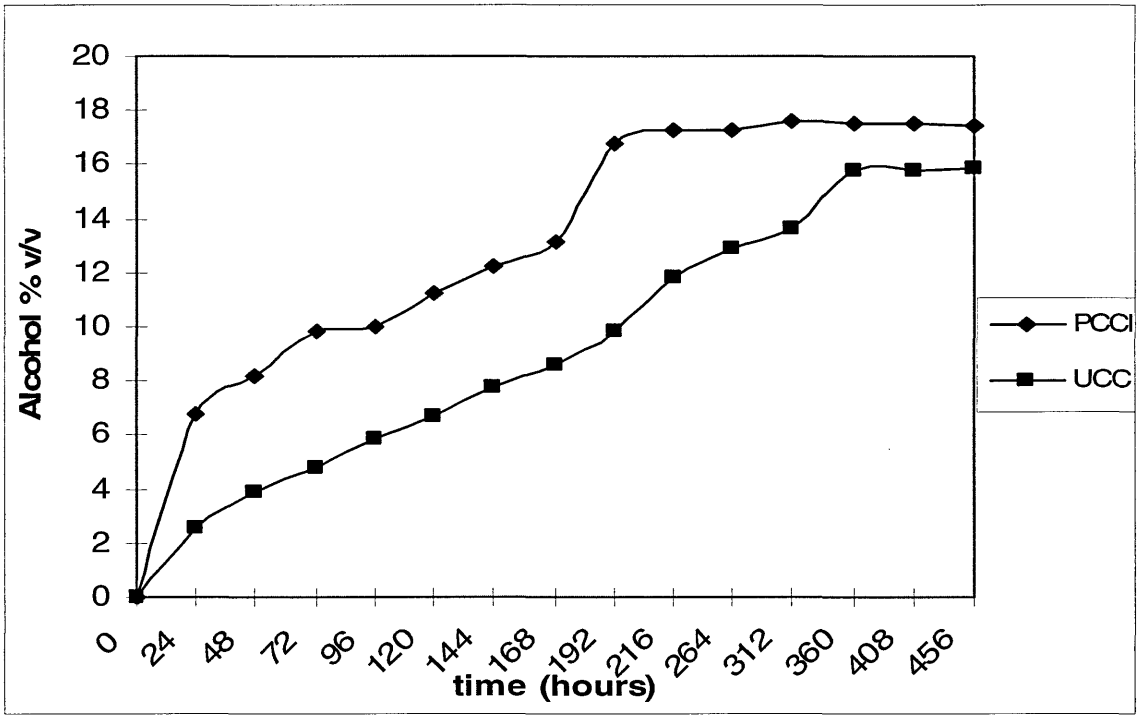


Figure 49 Alcohol production by salt pre-conditioned yeast during 2006 Chardonnay grape must fermentation
Fermentations were conducted with preconditioned yeast (Vitilevure Chardonnay) using 6% NaCl and growing cells for 16 hours prior to inoculation of 5000 L stainless steel tanks containing Chardonnay must. Must was clarified by pectinolytic enzymes and filtered before inoculation. Fermentation by unconditioned yeast cells is also demonstrated.

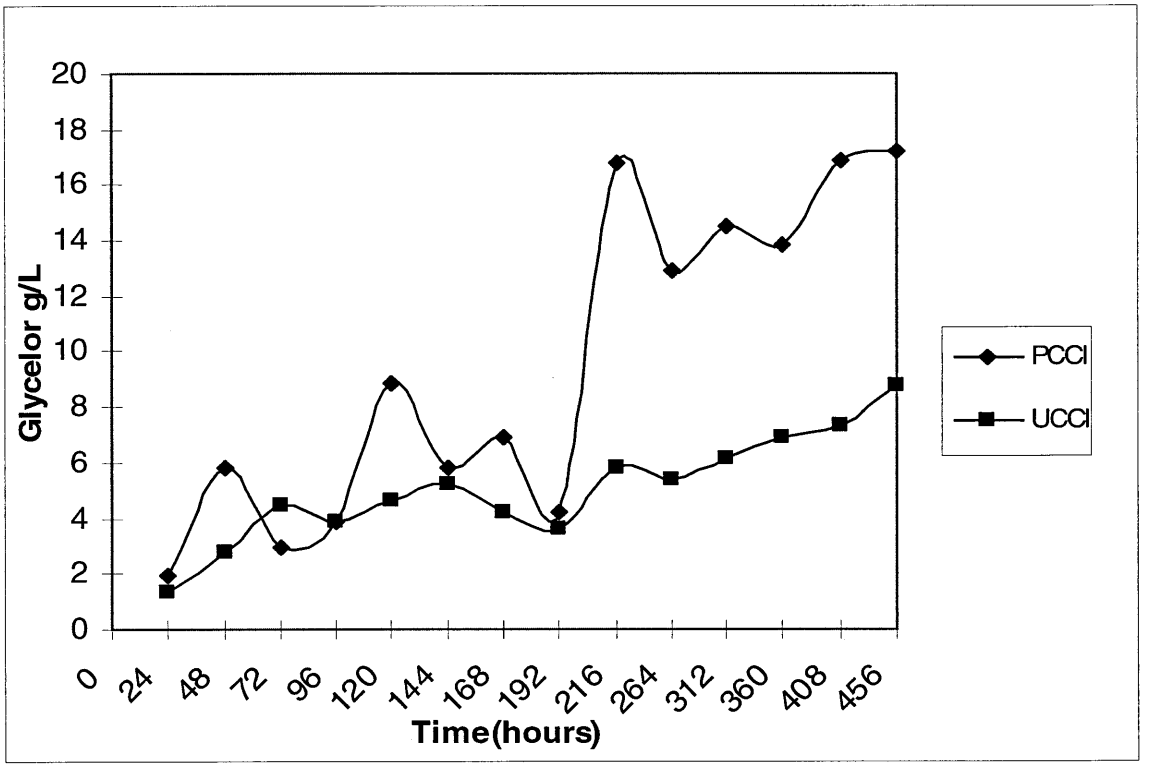


Figure 50 Glycerol production by salt pre-conditioned yeast during 2006 Chardonnay grape must fermentation
Fermentations were conducted with preconditioned yeast (Vitilevure Chardonnay) using 6% NaCl and growing cells for 16 hours prior to inoculation of 5000 L stainless steel tanks containing Chardonnay must. Must was clarified by pectinolytic enzymes and filtered before inoculation. Fermentation by unconditioned yeast cells is also demonstrated.

2007 Industrial fermentations

Figures 51-58 show the results of 2007 fermentations which were similar to previous years regarding sugar consumption, alcohol production and glycerol production, between salt-preconditioned and unconditioned cells. For salt-preconditioned cells, alcohol was higher by approximately 8% v/v; glycerol was 11 g/L higher; and residual sugars were 6 g/L higher.

During this year we additionally monitored: glucose and fructose consumption, volatile acidity, total acidity and pH. Both glucose and fructose were consumed quickly, especially for the first 24 hours of fermentation. Fructose levels remained high for the first 96 hours and between 96 and 120 hours we have a big decrease for both control and preconditioned cells fermentations. Pre-conditioned cells appeared to exhibit a slower rate of fructose consumption compared with unconditioned cells. Preferential utilization of glucose, before other sugars (like fructose) are metabolized is a well-known phenomenon in yeast. Total acidity and pH were almost the same for both of the fermentations but a big difference in volatile acidity were observed which at close was 0.56 g/L for the unconditioned cells and 0.2 g/L for the pre-conditioned cells.

Table 5 Fermentation performance of salt pre-conditioned (6% NaCl w/v) wine yeast (Vitilevure Chardonnay) in 2007 Chardonnay grape must. Fermentation performance (in 5000L vessels) was assessed by sugar consumption, alcohol and glycerol production. PCC represents pre-conditioned cells and UCC represents unconditioned cells.

Time (Hours)	Sugars g/L		Alcohol % v/v		Glycerol g/L	
	PCC	UCC	PCC	UCC	PCC	UCC
0	365	365	0	0	0	0
24	164.64	175.65	8.33	8.39	3.45	3.89
48	160.42	175.32	8.55	8.31	3.99	4.82
72	159.04	175.32	8.44	8.33	2.5	3.82
96	81.95	55.88	14.85	15.57	6.07	6.6
120	81.01	55.56	14.85	15.59	5.96	6.3
144	80.42	55.15	14.82	15.54	6.3	6.45
202	81.31	56.65	14.71	15.45	6.43	6.29
226	81.56	56.77	14.76	15.44	6.21	6.72
298	81.75	56.33	14.77	15.37	6.38	6.33
322	73.82	50.08	15.31	15.2	8.22	4.21
538	69.87	0.95	15.32	11.98	7.76	3.94
610	49.35	0.92	16.95	11.53	7.58	3.84
720	8.4	0.2	19.2	11.2	15.2	3.9

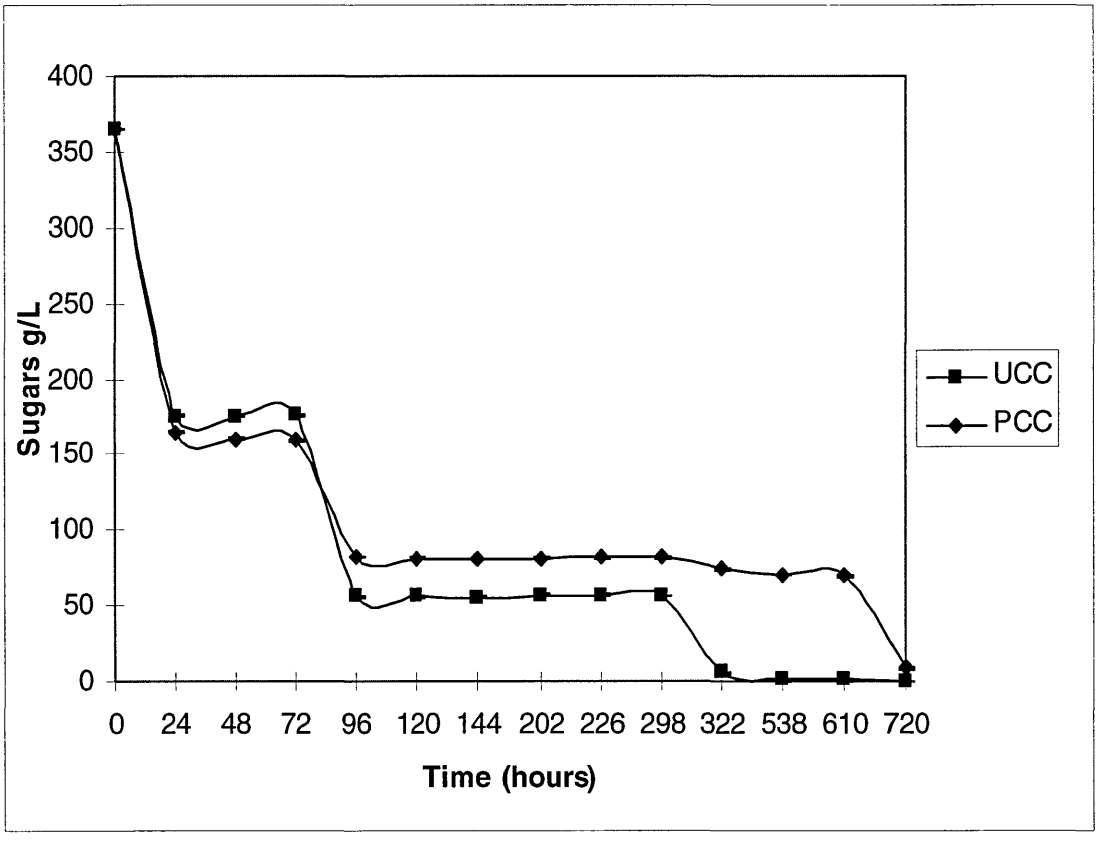


Figure 51 Residual sugars for salt pre-conditioned yeast during 2007 Chardonnay grape must fermentation
Fermentations were conducted with preconditioned yeast (Vitilevure Chardonnay) using 6% NaCl and growing cells for 16 hours prior to inoculation of 5000 L stainless steel tanks containing Chardonnay must. Must was clarified by pectinolytic enzymes and filtered before inoculation.
Fermentation by unconditioned yeast cells is also demonstrated.

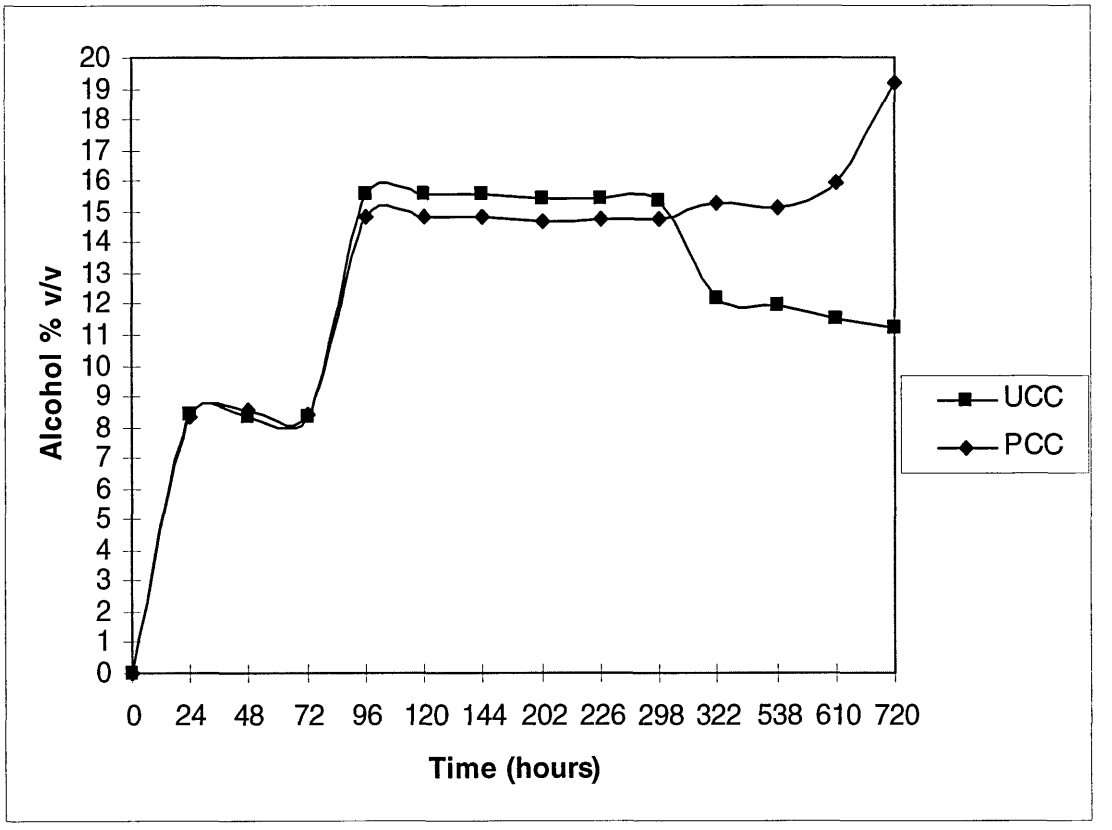


Figure 52 Alcohol production by salt pre-conditioned yeast during 2007 Chardonnay grape must fermentation
Fermentations were conducted with preconditioned yeast (Vitilevure Chardonnay) using 6% NaCl and growing cells for 16 hours prior to inoculation of 5000 L stainless steel tanks containing Chardonnay must. Must was clarified by pectinolytic enzymes and filtered before inoculation. Fermentation by unconditioned yeast cells is also demonstrated.

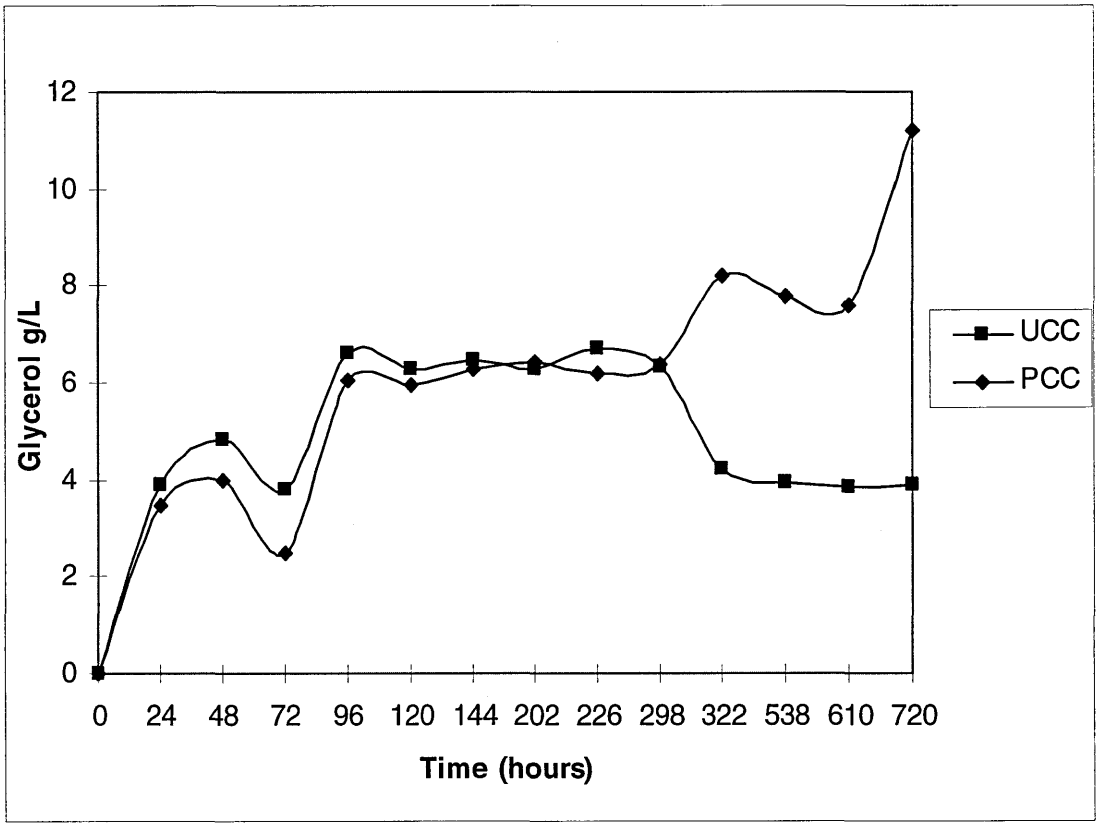


Figure 53 Glycerol production by salt pre-conditioned yeast during 2007 Chardonnay grape must fermentation
Fermentations were conducted with preconditioned yeast (Vitilevure Chardonnay) using 6% NaCl and growing cells for 16 hours prior to inoculation of 5000 L stainless steel tanks containing Chardonnay must. Must was clarified by pectinolytic enzymes and filtered before inoculation. Fermentation by unconditioned yeast cells is also demonstrated.

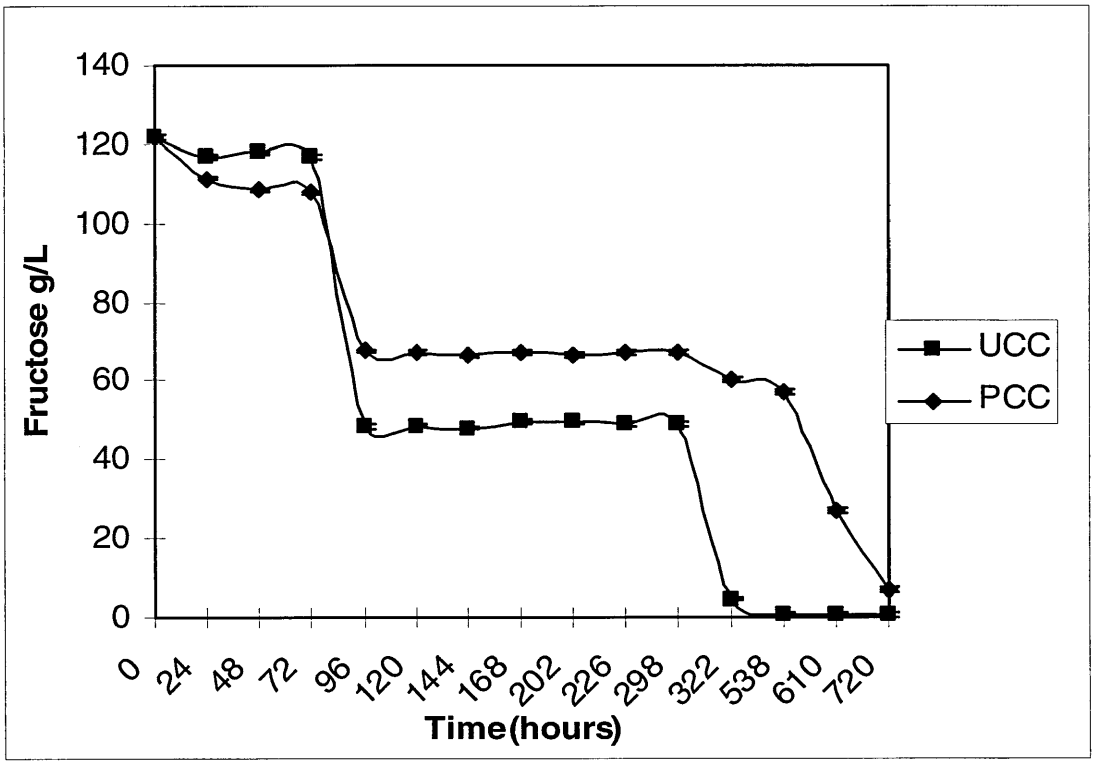


Figure 54 Fructose consumption by salt pre-conditioned yeast during 2007 Chardonnay grape must fermentation
Fermentations were conducted with preconditioned yeast (Vitilevure Chardonnay) using 6% NaCl and growing cells for 16 hours prior to inoculation of 5000 L stainless steel tanks containing Chardonnay must. Must was clarified by pectinolytic enzymes and filtered before inoculation. Fermentation by unconditioned yeast cells is also demonstrated.

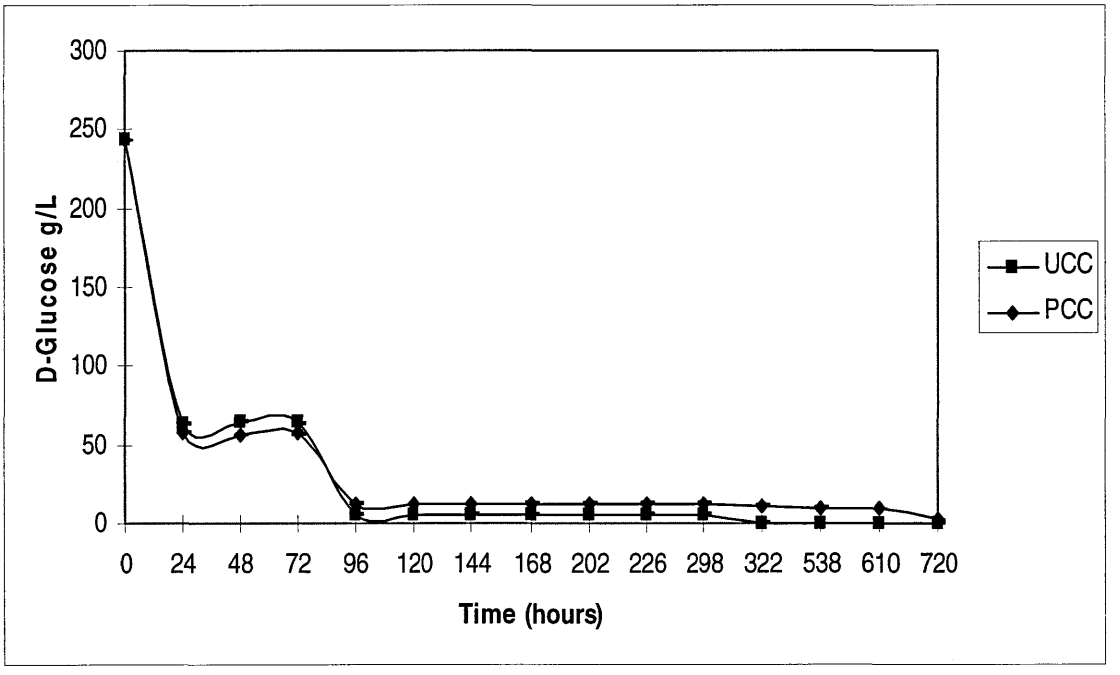


Figure 55 Glucose consumption by salt pre-conditioned yeast during 2007 Chardonnay grape must fermentation
Fermentations were conducted with preconditioned yeast (Vitilevure Chardonnay) using 6% NaCl and growing cells for 16 hours prior to inoculation of 5000 L stainless steel tanks containing Chardonnay must. Must was clarified by pectinolytic enzymes and filtered before inoculation. Fermentation by unconditioned yeast cells is also demonstrated.

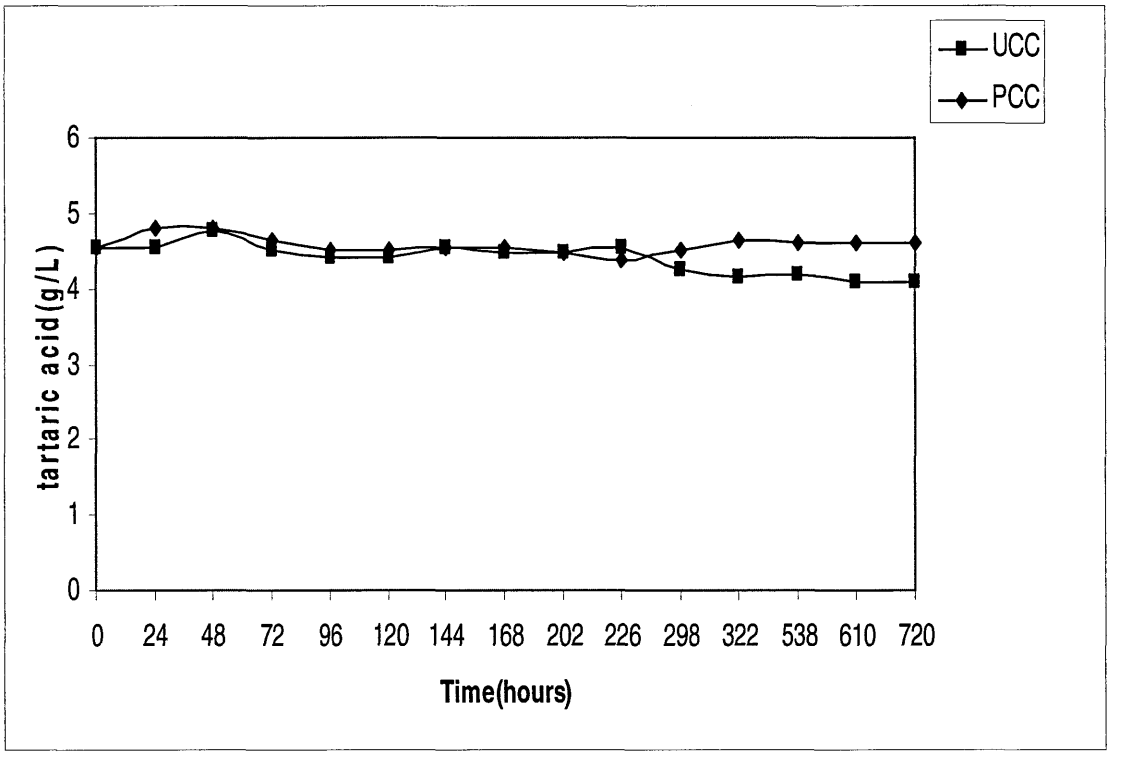


Figure 56 Total acidity in fermentations with salt pre-conditioned yeast in 2007 Chardonnay grape must
Fermentations were conducted with preconditioned yeast (Vitilevure Chardonnay) using 6% NaCl and growing cells for 16 hours prior to inoculation of 5000 L stainless steel tanks containing Chardonnay must. Must was clarified by pectinolytic enzymes and filtered before inoculation. Fermentation by unconditioned yeast cells is also demonstrated.

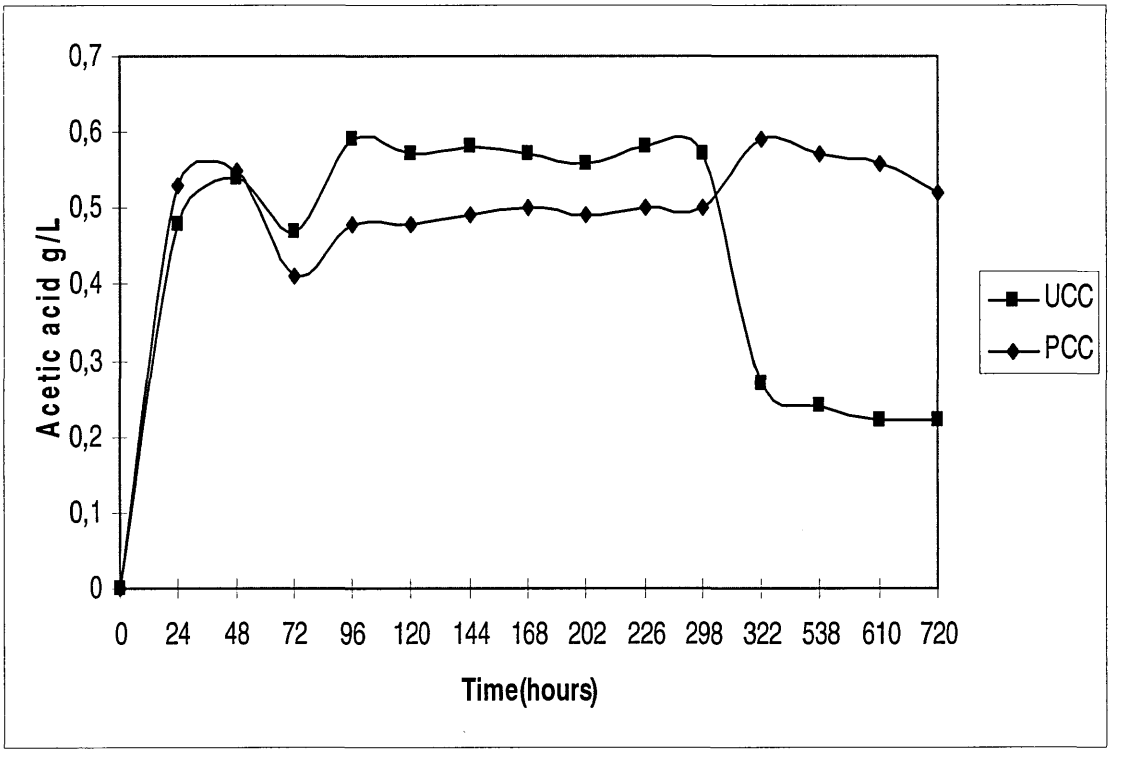


Figure 57 Volatile acidity in fermentations with salt pre-conditioned yeast in 2007 Chardonnay grape must
Fermentations were conducted with preconditioned yeast (Vitilevure Chardonnay) using 6% NaCl and growing cells for 16 hours prior to inoculation of 5000 L stainless steel tanks containing Chardonnay must. Must was clarified by pectinolytic enzymes and filtered before inoculation. Fermentation by unconditioned yeast cells is also demonstrated.

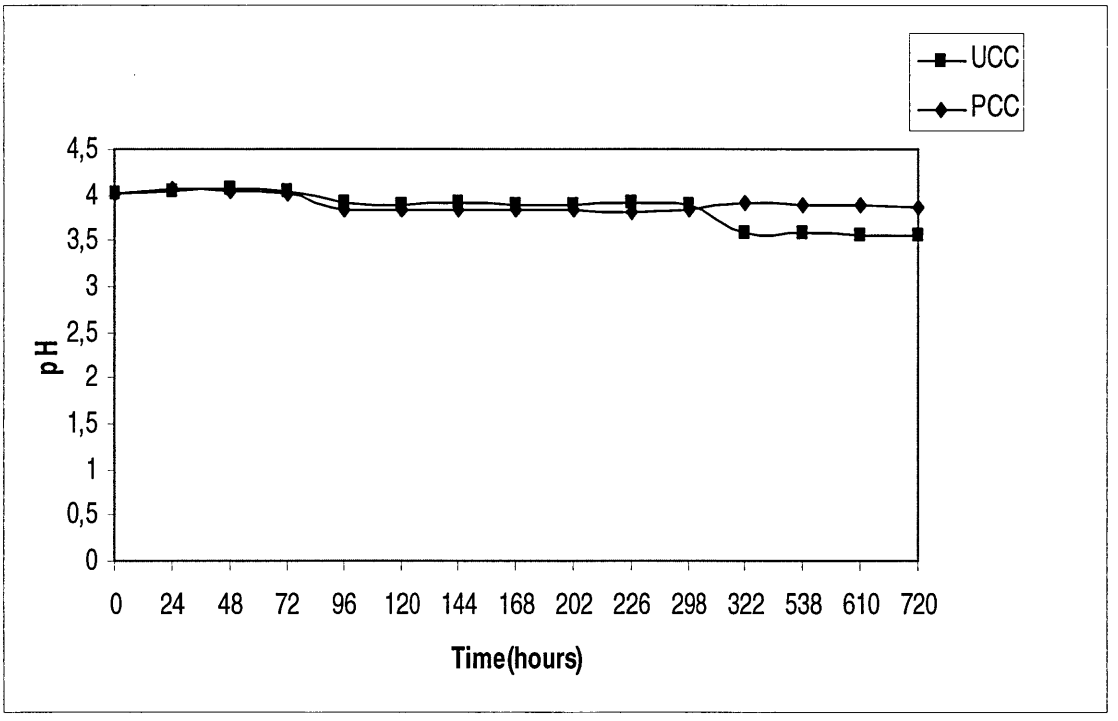


Figure 58 pH changes in fermentations with salt pre-conditioned yeast in 2007 Chardonnay grape must
Fermentations were conducted with preconditioned yeast (Vitilevure Chardonnay) using 6% NaCl and growing cells for 16 hours prior to inoculation of 5000 L stainless steel tanks containing Chardonnay must. Must was clarified by pectinolytic enzymes and filtered before inoculation. Fermentation by unconditioned yeast cells is also demonstrated.

2008 Industrial fermentations

During this year of grape harvest and experimentation we evaluated the fermentation ability of 3 industrial wine yeast strains of *S. cerevisiae* (*Vitilevure Chardonnay*, *Vitilevure KD* and *Vitilevure CSM*). Figures 59-64 demonstrate the basic characteristics of these 3 yeast strains regarding sugar consumption, alcohol production, volatile acidity, total acidity and glycerol production. As fermentation substrate, we have used Chardonnay and Merlot grape must with the same sugar concentration (306 g/L). It can be seen that strains produced almost the same alcohol around 16.5% vol. Total acidity and pH were almost the same but *Chardonnay* yeast fermentation produced more glycerol, the lowest volatile acidity and had the lowest residual sugars at the end of the fermentation compared with the other two wine yeast strains. This was similarly observed in the 2005 industrial fermentation and has the same benefits for wine characteristics as described above (see 2005 industrial scale fermentation).

Table 6 Fermentation performance of salt pre-conditioned (6% NaCl w/v) wine yeast (Vitilevure Chardonay, Vitilevure KD, Vitilevure CSM) in 2008 Chardonnay and Merlot grape must.
Fermentation performance (in 5000L vessels) was assessed by sugar consumption, alcohol and glycerol production. PCC represents preconditioned cells and UCC represents unconditioned cells.

Merlot fermentation with *Vitilevure KD*

Time (hours)	0	24	48	72	96	120	144	168	192	216	240	264
Ethanol % v/v	0	5.2	8.9	13.2	15.5	15.5	15.6	15.6	15.8	15.8	16.3	16.5
Total Acidity g/L of tartaric acid	5.5	5.2	5.3	5.2	5.1	5.3	5.1	5.2	4.9	5.1	4.9	5.1
Volatile Acidity g/L of acetic acid	0	0.23	0.35	0.56	0.69	0.71	0.71	0.71	0.71	0.70	0.71	0.75
pH	3.82	3.79	3.76	3.75	3.71	3.70	3.69	3.70	3.67	3.70	3.68	3.71
Reducing Sugar g/L	306	206	149	93	75.6	76.4	75.5	74.8	74.4	72.2	66.4	63.5
Glycerol g/L	0	3.2	4.8	5.3	6.5	6.6	6.4	6.5	6.7	6.6	6.6	6.7

Merlot Fermentation with *Vitilevure CSM*

Ethanol % v/v	0	6.3	9.1	13.8	13.1	13.0	13.1	13.0	13.1	13.9	14.2	16.3
Total Acidity g/L of tartaric acid	5.5	5.1	5.2	5.3	5.3	5.2	5.3	5.3	5.2	5.3	5.3	4.9
Volatile Acidity g/L of acetic acid	0	0.33	0.88	1.01	0.97	0.96	0.98	0.97	0.96	1.01	0.99	0.70
pH	3.82	3.72	3.75	3.78	3.83	3.81	3.82	3.82	3.82	3.77	3.77	3.69
Reducing Sugar g/L	306	202	145	103.7	117.5	117.7	116.0	117.2	116.6	105.8	101.7	65.5
Glycerol g/L	0	3.6	5.4	6.2	5.9	6.1	5.9	6.1	6.0	6.5	6.4	6.6

Chardonnay Fermentation with *Vitilevure Chardonnay*

Ethanol % v/v	0	6.2	8.9	13.8	14.1	14.6	15.7	15.8	16.0	16.1	16.3	16.8
Total Acidity g/L of tartaric acid	5.2	5.1	5.3	5.4	5.3	5.3	5.1	5.1	5.1	5.2	5.2	4.9
Volatile Acidity g/L of acetic acid	0	0.42	0.49	0.52	0.53	0.52	0.55	0.55	0.55	0.57	0.56	0.58
pH	3.9	3.83	3.84	3.84	3.86	3.88	3.92	3.93	3.95	4.01	3.99	4.04
Reducing Sugar g/L	306	204	148	67.0	61.3	50.7	37.8	36.1	32.0	29.4	27.4	20.2
Glycerol g/L	0	4.5	6.2	7.4	7.7	7.8	7.9	7.9	7.9	8.1	8.1	8.0

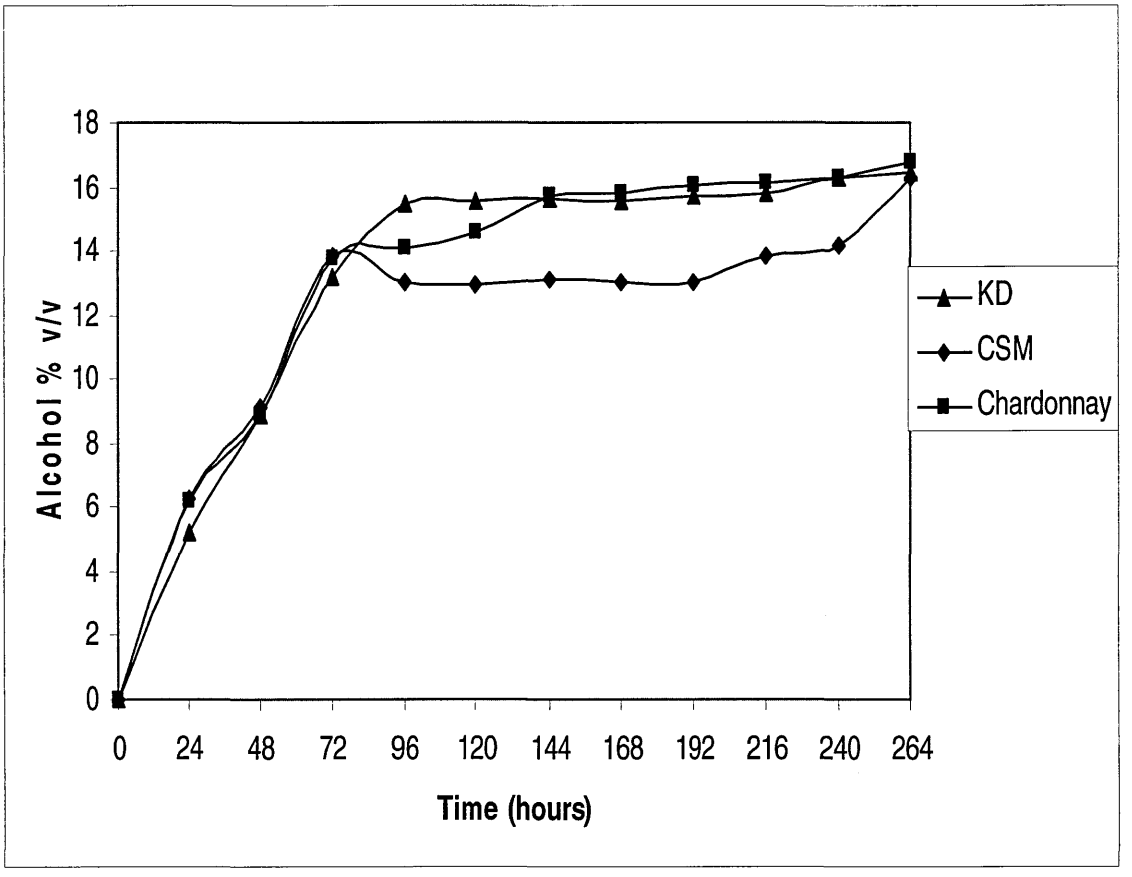


Figure 59 Alcohol production by three salt pre-conditioned wine yeasts during fermentation of 2008 Chardonnay and Merlot must
Fermentations were conducted with preconditioned yeasts (strains *Vitilevure Chardonnay, Vitilevure KD and Vitilevure SCM* from Martin Viallate, France) using 6% NaCl and growing cells for 24 hours prior to inoculation of 5000 L stainless steel tanks containing Chardonnay and Merlot must. Must was clarified by pectinolytic enzymes and filtered before inoculation.

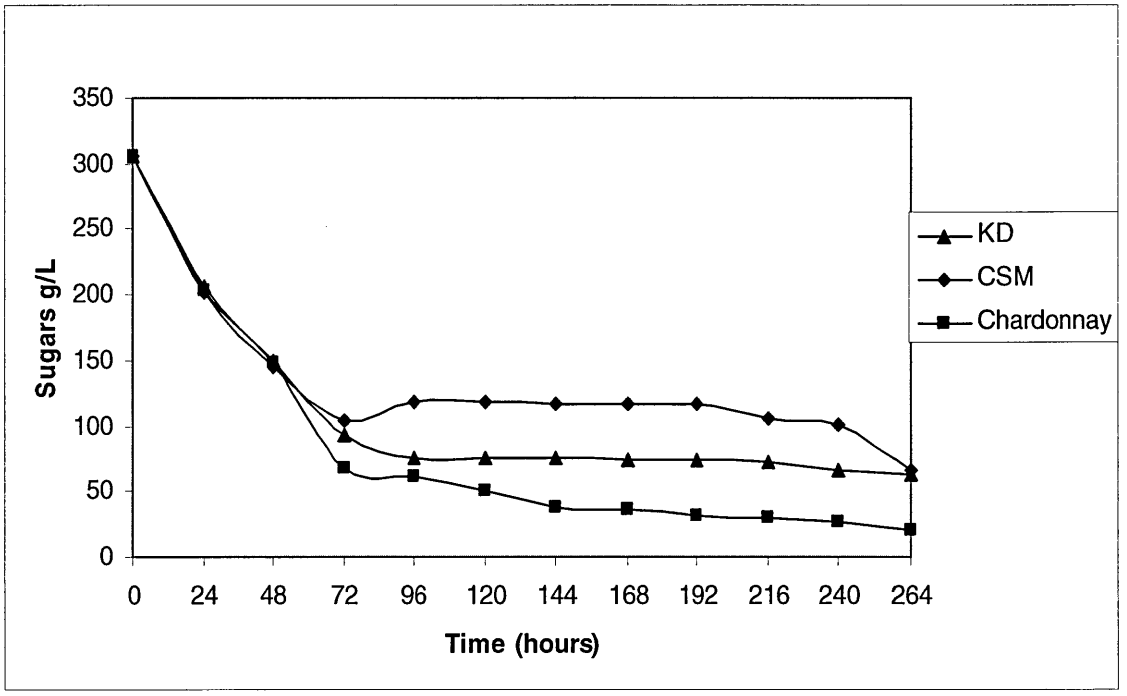


Figure 60 Sugar consumption by three salt pre-conditioned wine yeasts during fermentation of 2008 Chardonnay and Merlot must
Fermentations were conducted with preconditioned yeasts (strains *Vitilevure Chardonnay, Vitilevure KD and Vitilevure SCM* from Martin Viallate, France) using 6% NaCl and growing cells for 24 hours prior to inoculation of 5000 L stainless steel tanks containing Chardonnay and Merlot must. Must was clarified by pectinolytic enzymes and filtered before inoculation.

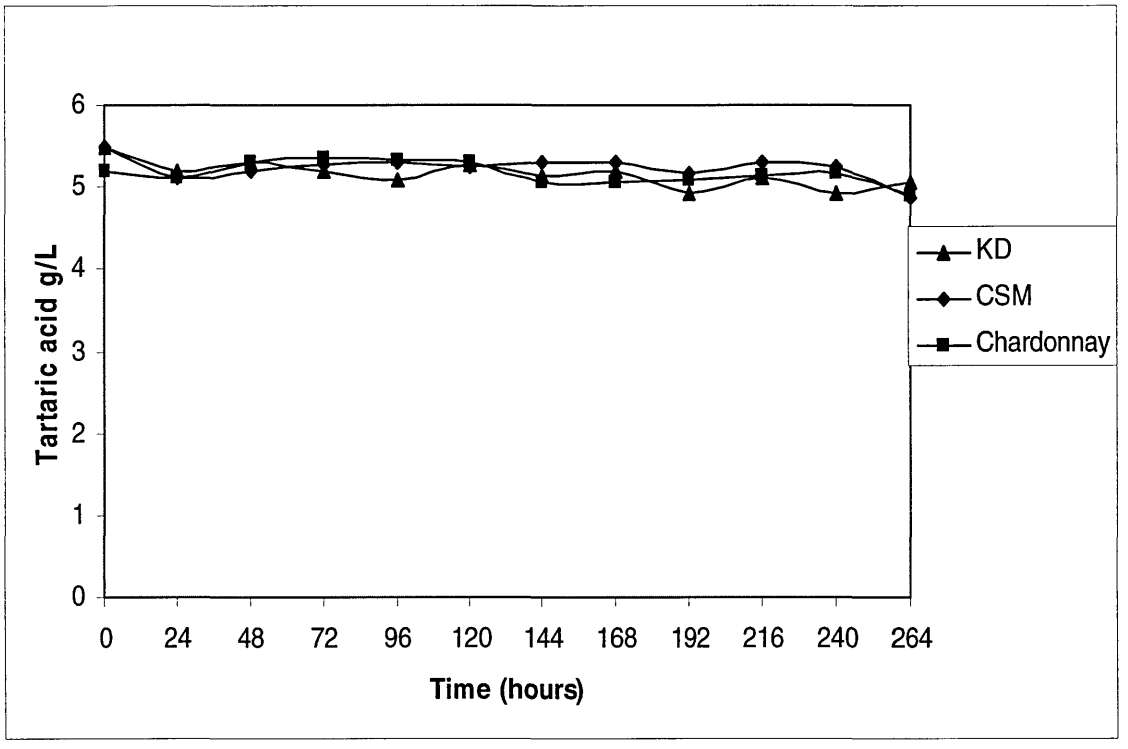


Figure 61 Total acidity during fermentation by three salt pre-conditioned wine yeasts of 2008 Chardonnay and Merlot must
Fermentations were conducted with preconditioned yeasts (strains *Vitilevure Chardonnay, Vitilevure KD and Vitilevure SCM* from Martin Viallate, France) using 6% NaCl and growing cells for 24 hours prior to inoculation of 5000 L stainless steel tanks containing Chardonnay and Merlot must. Must was clarified by pectynolitic enzymes and filtered before inoculation.

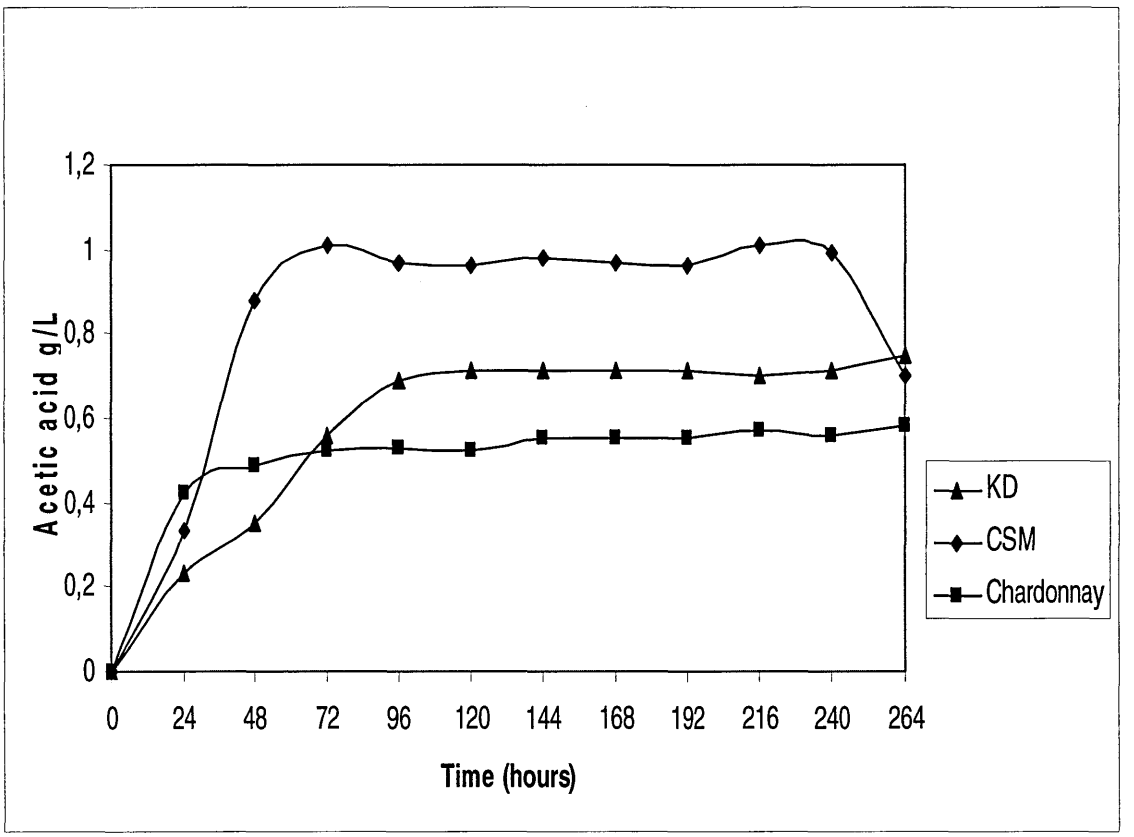


Figure 62 Volatile acidity during fermentation by three salt pre-conditioned wine yeasts of 2008 Chardonnay and Merlot must
Fermentations were conducted with preconditioned yeasts (strains *Vitilevure Chardonnay, Vitilevure KD and Vitilevure SCM* from Martin Viallate, France) using 6% NaCl and growing cells for 24 hours prior to inoculation of 5000 L stainless steel tanks containing Chardonnay and Merlot must. Must was clarified by pectynolitic enzymes and filtered before inoculation.

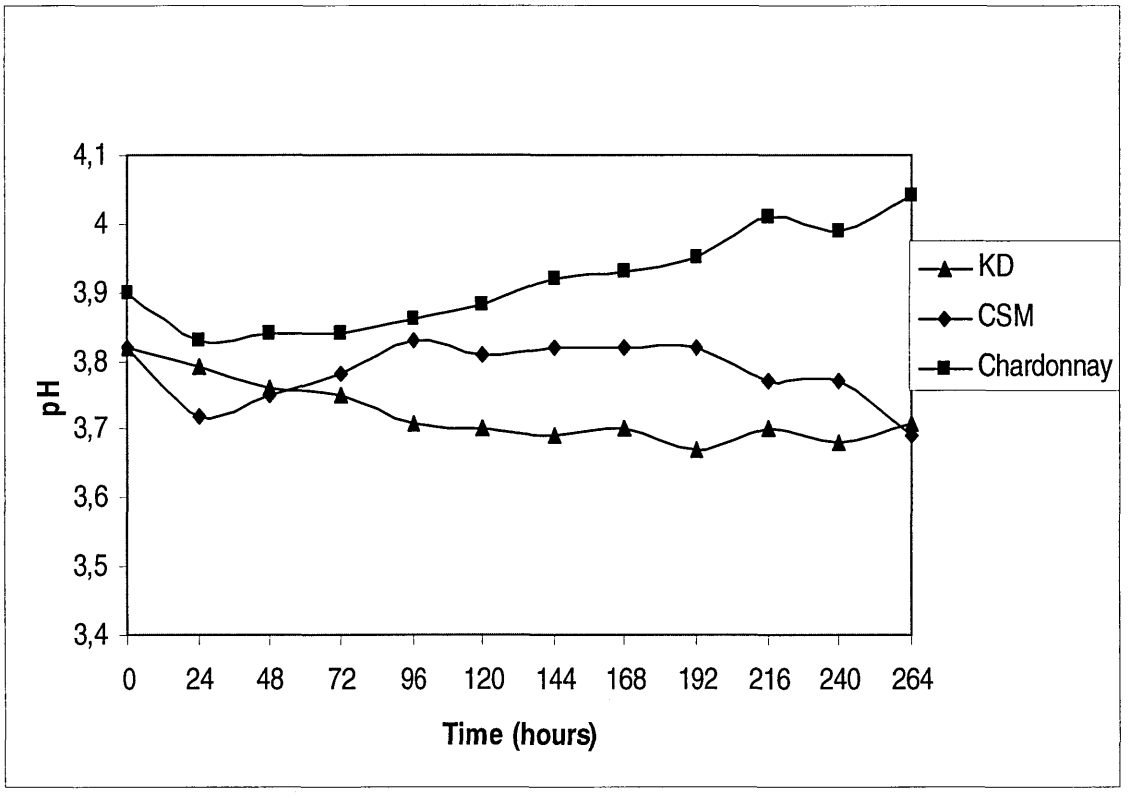


Figure 63 pH changes during fermentation by three salt pre-conditioned wine yeasts of 2008 Chardonnay and Merlot must
Fermentations were conducted with preconditioned yeasts (strains *Vitilevure Chardonnay, Vitilevure KD and Vitilevure SCM* from Martin Viallate, France) using 6% NaCl and growing cells for 24 hours prior to inoculation of 5000 L stainless steel tanks containing Chardonnay and Merlot must. Must was clarified by pectynolitic enzymes and filtered before inoculation.

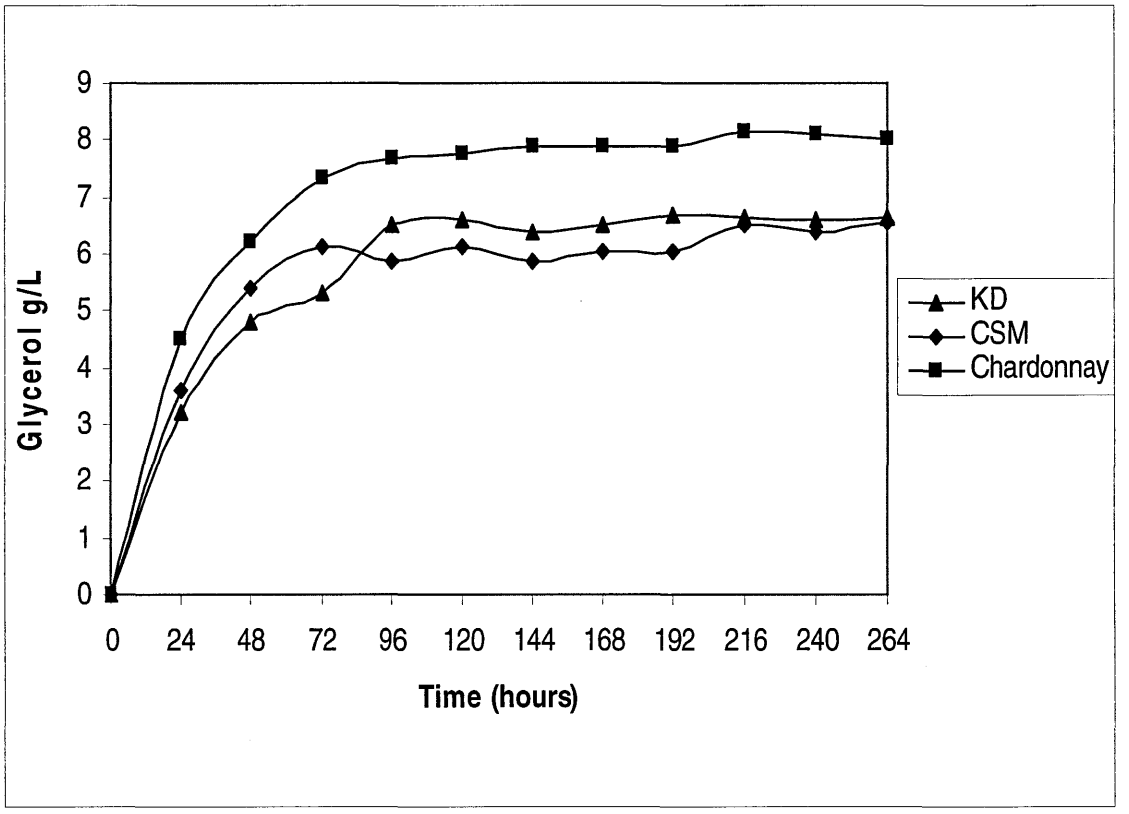


Figure 64 Glycerol production by three salt pre-conditioned wine yeasts during fermentation of 2008 Chardonnay and Merlot must
Fermentations were conducted with preconditioned yeasts (strains *Vitilevure Chardonnay, Vitilevure KD and Vitilevure SCM* from Martin Viallate, France) using 6% NaCl and growing cells for 24 hours prior to inoculation of 5000 L stainless steel tanks containing Chardonnay and Merlot must. Must was clarified by pectynolitic enzymes and filtered before inoculation.

The collective results from these industrial-scale fermentations will now be considered, by focusing on effects of ethanol toxicity and osmostress on yeast and their impact on winemaking processes.

It is well known that during alcoholic fermentation under anaerobic conditions, yeasts are affected by high sugar concentration stress during the early stages, and latterly by the continuously increasing alcohol concentrations, together with nutrient starvation. Increasing levels of ethanol during alcoholic fermentation inhibits glycolytic enzymes and biological processes, which are associated with membrane lipid composition (unsaturation of membrane lipids). During ethanol production at high levels it has been reported by Piper (1995) that leakage of oxygen occurs from cytochrome pigments. Incomplete reduction of molecular oxygen by the respiratory chain of the mitochondrion causes the production of an endogenous superoxide anion. This anion may cause severe oxidative damage to proteins, lipids and to DNA. Ethanol inhibits glucose, maltose, ammonium and amino acid uptake and causes leakage of potassium, amino acids and nucleotides from yeast cells. When yeasts are exposed to high ethanol levels the activity of H^+-ATPase (which is the major enzyme responsible for maintaining the electrochemical potential gradient across the plasma membrane), is dramatically stimulated. At the same time ethanol causes an alteration of the fatty acids and sterol composition, induced lipolysis of cellular phospholipids, increased ionic permeability and inhibition of nutrient uptake and hyperpolarization of the plasma membrane. During fermentation, ethanol inhibits cell division and cell viability and decreases yeast cell

volume (Vitor Costa *et al.*, 1993; Migozuchi and Hara, 1998; Peres and Laluce, 1998; and Walker, 1998).

It has been reported for some types of sweet wines like "Vino Cotto", an Italian sweet wine produced in central Italy, the Sauterne wines produced in France, and Ice wines produced in Canada, that yeast was exposed to osmotic stress caused by high sugar concentrations and that *S. cerevisiae* was tolerant to alcohol at 14% v/v (Tofalo *et al*,. 2009). During alcoholic fermentation for wine production, yeasts cells have to manage with different types of stresses as previously discussed in this thesis. According to research by Zuzuarregui and Olmo (2004), 10 genes (which were triggered during an alcoholic fermentation) were similarly induced upon heat shock, osmostress and ethanol exposure. Two of those genes are involved in glycerol biosynthesis, which is one of the most important stress response elements, during early stages of fermentation. High initial sugar concentration in grape juice can affect yeast growth by increasing the lag phase, decreasing the growth rate, decreasing the maximum cell population and decreasing the ethanol tolerance in later stages of fermentation (Lafon-Lafourcade,1983;Monk and Cowley, 1984; Nishino, *et al.*, 1985).

Other studies (van Vuren. *et al.,* 2003) have shown that about 589 genes were triggered during fermentations which had concentrations up to 40% w/v sugar. Additionally, an increase of acetic acid production has been reported together with an arrest in cell growth (Erasmus *et al*, 2004). In Japan, the effect of NaCl on yeast growth and fermentation ability has been studied indicating that fermentation activity increased 20 to 30% at sucrose concentrations of 30% w/v (Kawai *et a*l,. 1999). It is well established that high sugar

concentration and ethanol inhibit the growth and viability of the cells during an alcoholic fermentation but how can some cells survive under those extreme conditions of osmotic stress?

Regarding the osmotic stress caused by sugar concentrations higher than 30% w/v and by alcohol, the response of *Sacchromyces cerevisiae* is primary based on the HOG pathway and the accumulation of intracellular glycerol and trehalose (see sections and 1.3.2 &1.3.3). In addition to the HOG pathway (see section 1.4), the RAS-CAMP PKA pathway is involved in regulating cell growth, carbon storage and stress response. Genes that are involved in the biosynthetic and dissimilatory pathways for glycerol are *GPD*1, *GPP*2, *GCY*1 and *DAK*1; for trehalose are *TPS*1, *TPS*3, *TSL*1, *PGM*2, *TPS*2, *NTH*1 and *ATH*1; and for glycogen *GSY*1, *GSY*2, *GLC*3 and *GPH*1 are up-regulated by high sugar concentrations but may also be the key genes induced for enabling yeast cells to respond to salt stress caused by NaCl.

Ethanol triggers the same mechanism as the HOG pathway and previous workers (Alexandre *et al.*, 2001) have found a series of genes associated with cell response and cell tolerance to ethanol stress. These genes (GPD1, *HOR*2, *DAK*1, *and SRE*3*)* are up-regulated during ethanol exposure. Additionally, genes such as *GSY*1, *GSY*2, *GCY*1 and *ATH*1 are up-regulated during ethanol stress. The same genes in addition to *HSP*104 and *HSP*12 are triggered under osmotic stress conditions caused by NaCl. Therefore, yeast cells have a common mechanism to manage stresses such as osmotic stress, ethanol stress and sugar stress (see section 1.4.2).

D'Amore and Stewart (1987) Koukou *et al.* (1990), Mackenzie et al., (1988) and Walker-Caprioglio *et al*, (1990) have shown that under those extreme stress conditions some cells can survive via the molecular and genetic mechanism that we have already described (see section 1.4).

Since wine yeasts have to cope with different kinds of stresses and pH is one of the most important factors that affect the fermentation ability of the cells. pH of wine must is between 3 and 4 and there is some evidence that the yeast growth and fermentation ability are decreased when the initial pH is low (Ough, 1966; Heard and Fleet, 1988).

Regarding the present industrial-scale trials, and laboratory-scale research findings, it can be concluded that NaCl pre-conditioned cells have acquired an ability to tolerate high concentrations of sugars and produce high amounts of alcohol in comparison with non-preconditioned cells. In addition, the production of glycerol was high and that is very important for wine product quality including "mouth taste" and "wine body". The alcohol yield for preconditioned cells was higher than the unconditioned cells throughout the four years' winemaking experiments.

Fermentation ability and sugar consumption appeared unaffected and during the first 24 hours sugar consumption was at its highest. Even the low pH had little influence on fermentability and ethanol productivity. The total sugar at the end of the fermentation, for the four years of experiments, was low and the alcohol was at very high levels. Especially for the industrial yeast strains of Vitilevure Chardonnay,

Vitilevure KD and Vitilevure CSM was higher than the expected maximum rate of alcohol production (information provided by the producing company, Martin Viallate, France). More specifically for the Vitilevure Chardonnay yeast strain, the highest alcohol content was achieved at 13.5% v/v, for Vitilevure KD 14.5% v/v and for Vitilevure CSM 14.0% v/v. Results for the three yeasts strains were: Vitilevure Chardonnay 17.4% v/v during 2006, 19.2% v/v during 2007 and 16.4 % v/v for 2008. Vitilevure KD reached 16.7% v/v and Vitilevure SCM reached 16.2% v/v. This indicates that we have an increased alcohol productivity and alcohol tolerance for all yeast strains during the years of experimentation. Additionally, yeasts displayed evidence of enhanced tolerance to high sugar concentrations, better adaptation to low pH levels and increased sugar consumption especially for the first 24-48 hours of fermentation.

To summarize, industrial scale experiments lead to a conclusion that osmotic pre-stress due to NaCl-preconditioning enabled yeast cells to ferment sugars at high concentrations, with high yields of alcohol produced. As previously discussed, the likely mechanism for this phenomenon lies at the level of elevated stress gene expression, especially linked to increases in the intracellular concentration of stress-tolerance metabolites such as glycerol and trehalose.

This has implications for winemaking. For example, winemakers occasionally need to cope with stuck and sluggish fermentation problems. "Stuck fermentation" refers to the premature termination of fermentation before all but trace amounts of fermentable sugars have

been metabolized. Both stuck and sluggish fermentations have been a problem since the early years of winemaking. Their occurrence in the past usually was attributed to overheating during fermentation. In the absence of adequate cooling, fruit harvested and fermented under hot conditions can readily overheat and fermentations become stuck. The resulting wines are high in residual sugar, making them particularly susceptible to microbial spoilage. Instability is increased further if the grapes are low in acidity, high in pH, or both. The extensive use of temperature control during fermentation has essentially eliminated overheating as a significant factor in stuck fermentations. The desire to accentuate the fresh, fruity character of white wines has encouraged the use of cool temperatures. This can limit yeast growth and potentially favour microbial contaminants that further retard growth. The osmotic effect of high sugar concentrations can also partially plasmolyze yeast cells, resulting in slow or incomplete fermentation. In addition, over-mature grapes may have an unusually low glucose:fructose ratio. This has been correlated with stuck fermentation in Switzerland (Schütz and Gafner, 1993).

The problems that cause stuck fermentations have been divided into four basic categories (Bisson and Butzke., 2000):

1) low initiation (eventually becoming normal),

2) continuously sluggish,

3) typical initiation, but becoming sluggish and

4) normal initiation but abrupt termination.

Comparing sugar consumption, temperature, nutrient profiles, and

records of procedures used with those from past fermentations, often provides early indications of potential problems and their possible quick resolution. Once fermentation has stopped, re-initiation is more complicated. When stuck fermentation occurs, successful re-initiation usually requires incremental re-inoculation with special yeast strains (obtained commercially), following racking off from settled lees (see Bisson and Butzke, 2000). The special strains usually possess high ethanol tolerance, as well as the ability to utilize fructose (the sugar whose proportion increases during fermentation). The addition of nutrients (if deficient), yeast "hulls" (cell wall preparations used to remove toxic fatty acids), must aeration, and adjustment of the fermentation temperature (if necessary) usually achieves successful re-fermentation.

During the last year (2008) of our industrial scale experiments, a stuck fermentation of a 12000L tank containing must from Syrah grape variety occurred (see Fig 65). The stuck fermentation was determined when the analyses of residual sugars was the same (10.6 g/L glucose) for a time period of 5 days. For secondary fermentation and for the re-inoculation we followed the procedure of inoculum preparation as previously described (Materials & Methods section 2.2). As indicated in Figures 66 to 68, this resulted in an increased alcohol production from 13,6% v/v to 14 % v/v and, at the same time, glucose concentration decreased under the limit of 3 g/L (which is the limit of dry red wines). The volatile acidity decreased to a level of 0.36 g/L of acetic acid and the total acidity increased close to 7 g/L of tartaric acid. The important

point to be made here is that in this procedure we have not use any alcohol tolerant yeast strain but the inoculation was made with a preconditioned *Vitilevure Chardonnay* strain.

The following results (Figures 65-68) indicate that the preconditioning of wine yeast to salt for a specific time can make the particular yeast strain alcohol tolerant and without resulting in problems regarding sugar utilisation and volatile productivity.

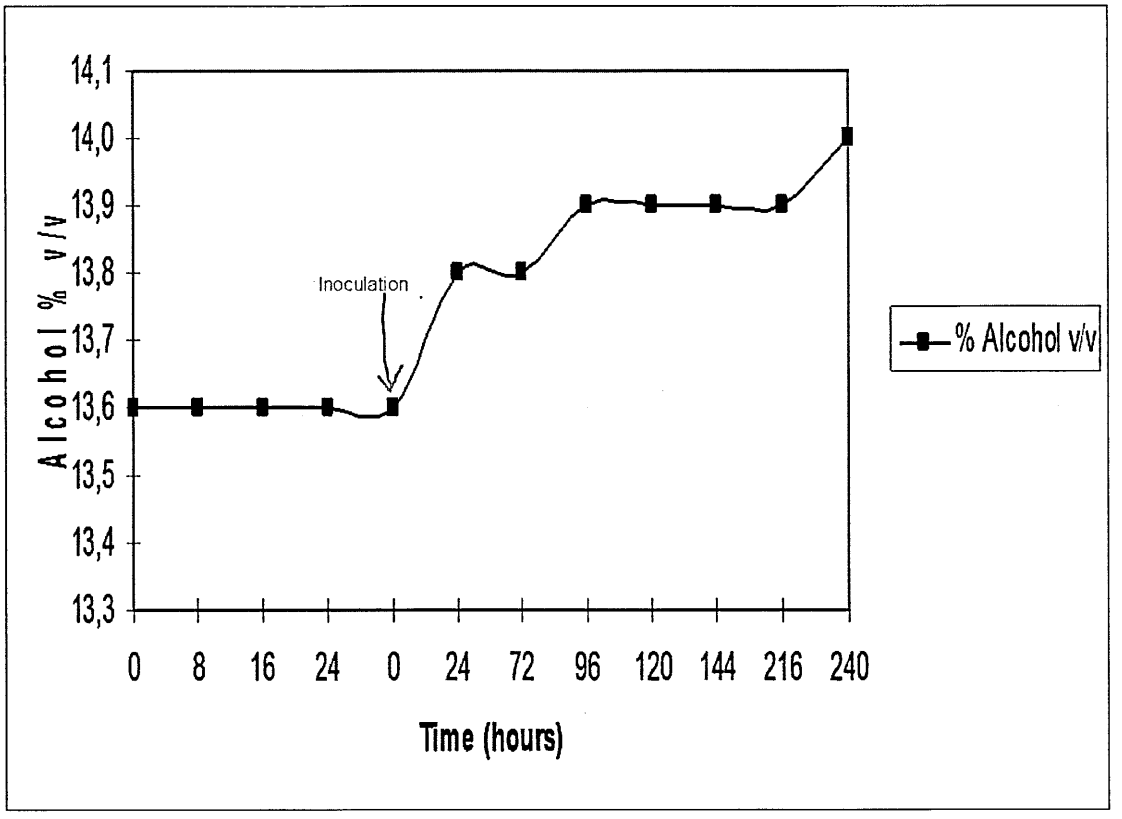


Figure 65 Alcohol production for re-inoculated stuck fermentation of Syrah wine using salt pre-conditioned yeast
Salt preconditioning was achieved using 6% w/v NaCl and growing cells for 16 hours. Yeast strain *Vitilevure Chardonnay* (strain from Anchor, S.Africa) was used for re-inoculation of 12000L stainless steel tanks containing Syrah wine. Wine was filtered before inoculation.

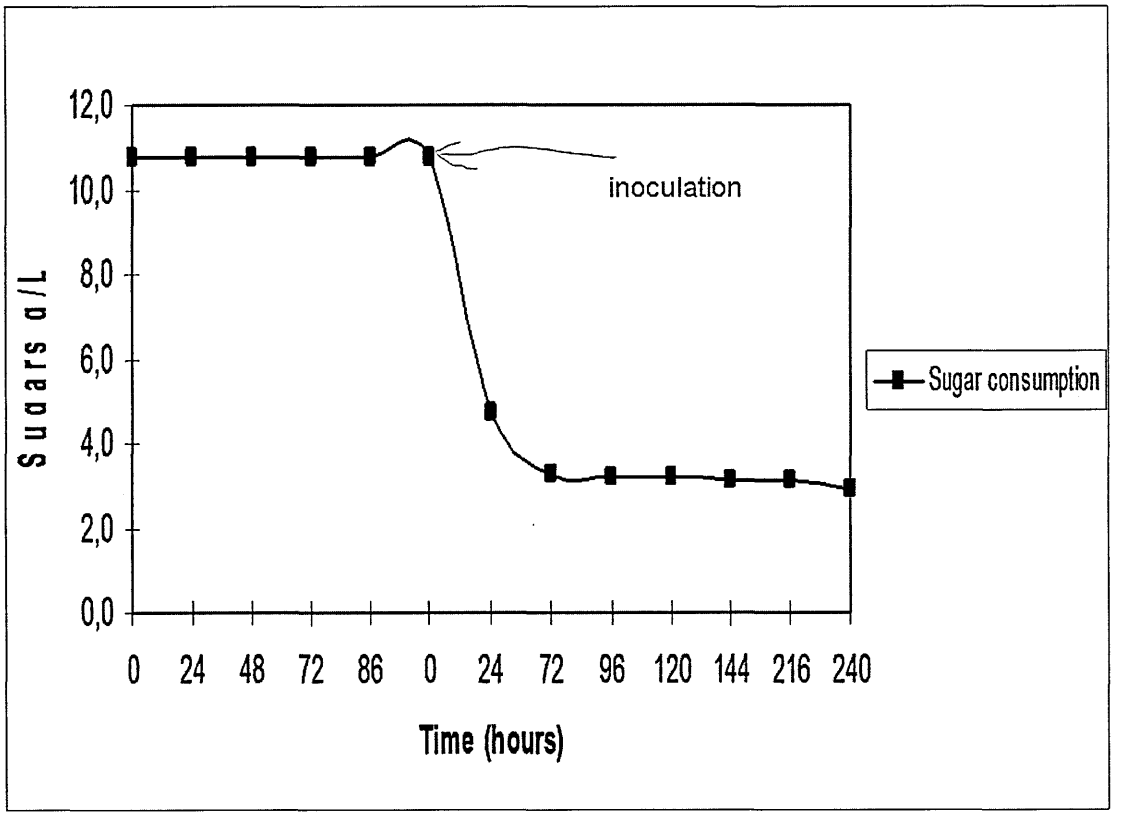


Figure 66 Sugar consumption for re-inoculated stuck fermentation of Syrah wine using salt pre-conditioned yeast
Salt preconditioning was achieved using 6% w/v NaCl and growing cells for 16 hours. Yeast strain *Vitilevure Chardonnay* (strain from Anchor, S.Africa) was used for re inoculation of 12000L stainless steel tanks containing Syrah wine. Wine was filtered before inoculation.

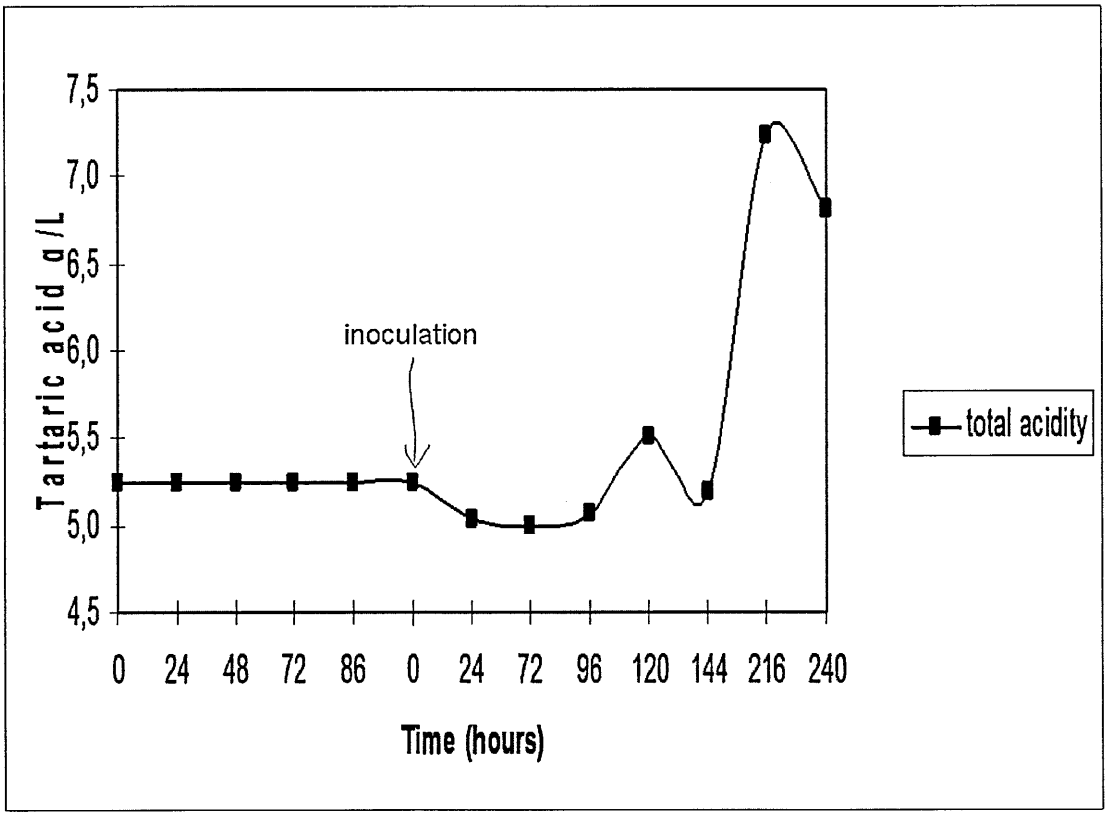


Figure 67 Total acidity for re-inoculated stuck fermentation of Syrah wine using salt pre-conditioned yeast
Salt preconditioning was achieved using 6% w/v NaCl and growing cells for 16 hours. Yeast strain *Vitilevure Chardonnay* (strain from Anchor, S.Africa) was used for re inoculation of 12000L stainless steel tanks containing Syrah wine. Wine was filtered before inoculation.

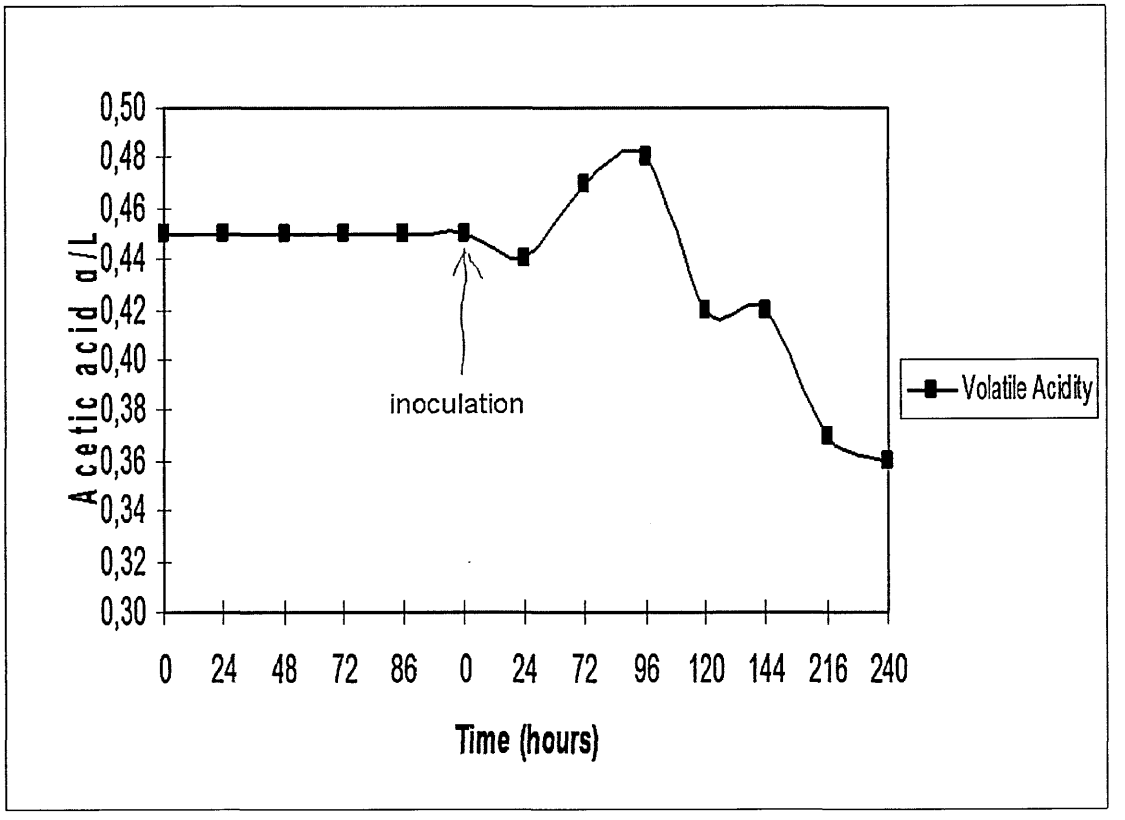


Figure 68 Volatile acidity for re-inoculated stuck fermentation of Syrah wine using salt pre-conditioned yeast
Salt preconditioning was achieved using 6% w/v NaCl and growing cells for 16 hours. Yeast strain *Vitilevure Chardonnay* (strain from Anchor, S.Afrika) was used for re inoculation of 12000L stainless steel tanks containing Syrah wine. Wine was filtered before inoculation.

3.5 Studies with salt-preconditioned wine yeast in a specialised fermentation system

In this section we investigated the fermentative behavior of salt-preconditioned yeast cells (*S.cerevisiae* Vitilevure SCM strain) using a specialized fermentation system which was previously designed by TEI (Technological Educational Institute) of Athens, Department of Enology (Nerantzis, Logothetis and Loziou 1995). The system has been named WITY (**W**ine **I**mmobilization **T**ower **Y**east) system and is a modular tower fermentation system designed to evaluate yeast strains especially those originating from the wine industry. In previous work, this system was used to describe the differences in yeast performance cultivated in batch and continuous modes during the process of wine production (Nerantzis *et al.*, 1995; Nerantzis and Logothetis., 2001). Most experimental fermentations were conducted using the wine yeast strain, Vin13. It was therefore deemed appropriate to investigate the behaviour of another yeast strain (Vitilevure SCM) which has been used in both laboratory and industrial scale experiments. Monitoring of five basic wine fermentation parameters (alcohol, residual sugars, volatile acidity, pH and glycerol) we attempted to evaluate the performance of this specific winemaking yeast strain regarding fermentation process improvement and product quality.

Additionally the WITY system possesses several advantages over similar systems, such as:

- efficient control of fermentation,
- high fermentable productivity,
- control of product quality,

- ability to use different yeast strains, and
- ability to valorize product characteristics.

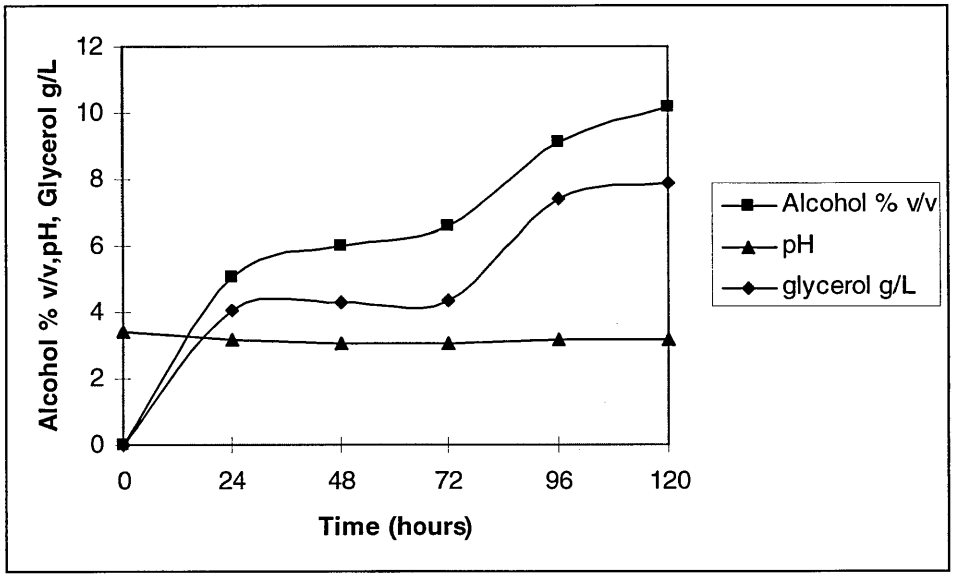
Additionally, yeasts in such a system exhibit shorter lag phases, are protected from sulphur dioxide, and produce lower volatile acidity and moderate increases in total acidity.

The main core of the WITY system is the immobilization of yeast. Recently, the application of immobilized biocatalysts in ethanol fermentation has been the subject of research in several laboratories (Chi-Bun Ching, *et al.,* 1990; Wada and Chibata, 1981). Cell immobilization can be achieved by self-aggregation, entrapment, containment behind a barrier, or adsorption/attachment (Bucke, *et al.,* 1979; Kierstan and Bucke, 1977). Entrapment in beads of calcium alginate gel is one of the most widely used techniques for immobilizing living microbial and animal cells (Gashin, 1995). In particular, immobilization of viable microbial cells, such as yeast cells, has gained much attention in recent years (Thomas and Gilson 1995). Immobilization is known to have many beneficial attributes like cell protection from ethanol toxicity, substrate inhibition and from low temperatures. Iimmobilization of yeast allows the use of high biomass concentration. High biomass concentration is known to increase product yield by reducing the total fermentation time, and has found practical applications in various processes (Gashin,1995). A number of reactor designs employing immobilized whole cells for continuous fermentation have been proposed (Nerantzis and Logothetis, 2001; Nerantzis *et al.,*

1995; Chi-Bun Ching, *et al.*, 1990).

In Figure 69 we demonstrate the results of a continuous mode fermentation using salt-preconditioned wine yeast for 120 hours using as media concentrated grape must diluted to 200 and 300 g/L sugar concentration. For the first 72 hours the system was introduced with medium at 200 g/L sugars and then continuously with medium containing 300g/L sugars.

A.



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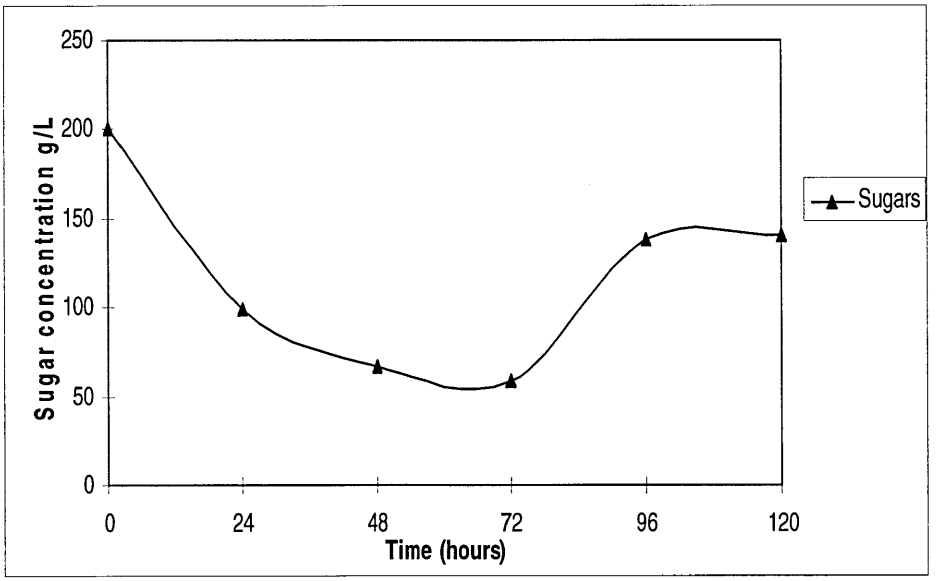

C.

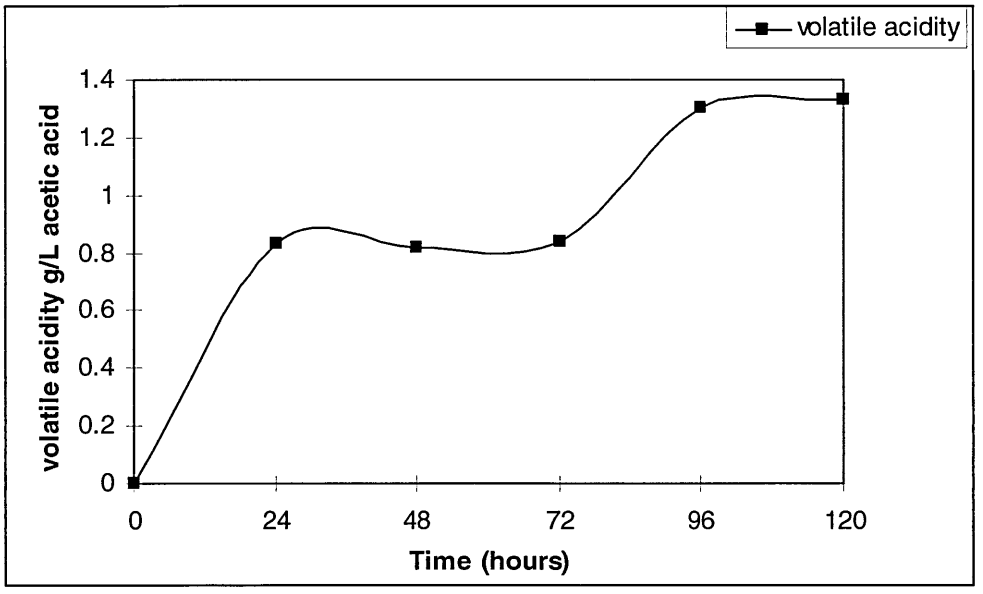

Figure 69 Fermentation performance of salt pre-conditioned wine yeast in a specialized immobilized fermentor (the WITY system)
Cells of wine yeast strain *S.cerevisiae* Vitilevure CSM were preconditioned with 6% w/v NaCl as described previously and allowed to establish in a WITY system tower fermentor with grape must as fermentation medium (see text for details).
A. Alcohol and glycerol production, and pH during the fermentation.
B. Sugar concentration during the fermentation
C. Volatile acidity during the fermentation

Figure 69 shows that during continuous immobilised fermentation in the WITY system, yeast produces alcohol during the period from 72 to 120 hours when the feeding of the system was made by medium which contained 300 g/L sugars. Glycerol production paralleled that of ethanol production and pH remained almost constant. Volatile acidity increased to 1.33 g/L of acetic acid and this represents a negative result of such a fermentation system (undesirable wine characteristic). Sugar concentration decreased for the first 72 hours and increased for the next

48 hours. This was due to the fact that at 72 hours, the sugar concentration of the medium was changed from 200 to 300 g/L. However, between 96 and 120 hours, this increase of sugar concentration was small (approximately 1.8 g/L) while at the same time the alcohol production increased by approximately 1% v/v. The same phenomenon occurred and between 48 and 72 hours when the increase of alcohol was approximately 1.2% v/v. This observation perhaps indicates that the system at this stage entered into in a steady state condition.

In general, and regarding the philosophy of the potential of WITY system for modern wine making, we can say that an immobilised yeast system like WITY can have valuable research and industrial uses (see Appendix 5). For example, it can be employed in the evaluation of wine yeasts regarding cellular preconditioning with salt and the impact of such novel treatments on product quality improvement.

4. CONCLUDING DISCUSSION

The idea of this research was born from Ancient Greece, and especially from the fermented foods for which they usually used high concentrations of salt and vinegar.

It is well known that in the Black Sea and in the areas where salt was collected from the sea there exists some species of halotolerant yeast and fungi. For example, the yeast *Debaromyces hansenii* is salt-tolerant and has been a subject of research for several years now.

The developed mechanisms of both yeast and the Ancient Greeks to produce fermented foods such as a fish sauce (Woods, 1998) under high concentrations of salt drove me to investigate the behaviour of some "commercial" wine yeasts under low and high concentrations of NaCl.

The results of the present work show that *S.cerevisiae* yeast cells represent a limited growth under osmotic stress due to NaCl. Non-*Saccharomyces* yeast strains that I have studied exhibit the same behaviour under salt stress conditions.

Regarding cellular viability, all yeast strains surprisingly retained, especially under high concentrations of NaCl. As previously discussed (see Introduction 1.3), under osmotic stress conditions cell defense mechanisms are triggered - the cell membrane composition changes and compatible solutes (glycerol and trehalose) are produced that will give the cells the ability to survive for long time periods under those extreme conditions (see Figure 25, Appendix 1,4 and 6).

For winemaking, one of the two compatible solutes that is produced,

glycerol, plays an important role in dictating wine quality and especially mouthful. The high production of glycerol in pre-conditioned cells as demonstrated in industrial scale experiments provides evidence that this can be translated to practical enology. Additionally, with the ability of pre-conditioned cells to metabolize high sugar concentrations, with low residual sugar levels, provides assurance to winemakers striving to conduct full conversion of grape sugars to alcohol. High production of alcohol can also be very beneficial in production of certain sweet wines.

The Chardonnay wine which was produced using salt pre-conditioned yeast during the 2006 grape harvest in Greece took a commended award at the International Wine Challenge 2007 in London. This provides further support to the benefits of the approaches adopted in this thesis to the wine industry. See Appendix 2.

A second wine which was produced during the year of 2008 with pre-conditioned yeast cells took a commended award at the International wine Challenge 2009 in London. See Appendix 3. Note that the results of the production of this specific wine are not included in this thesis.

The WITY system employed represents a system for continuous alcohol production using immobilized yeast, and has demonstrated potential in wine fermentations. Results presented have shown that around 10% v/v alcohol can be produced using preconditioned yeast in 120 hours in a continuous mode.

Further research with additional industrial yeast strains (eg. brewing and bioethanol yeasts) will verify the applicability of NaCl-

conditioning in other yeast biotechnologies. Potential (non-GM) applications of the research presented in this thesis are envisaged in the brewing and distilling industries. In that regard, the benefits of salt-preconditioning yeast cells can be summarized in the model below:

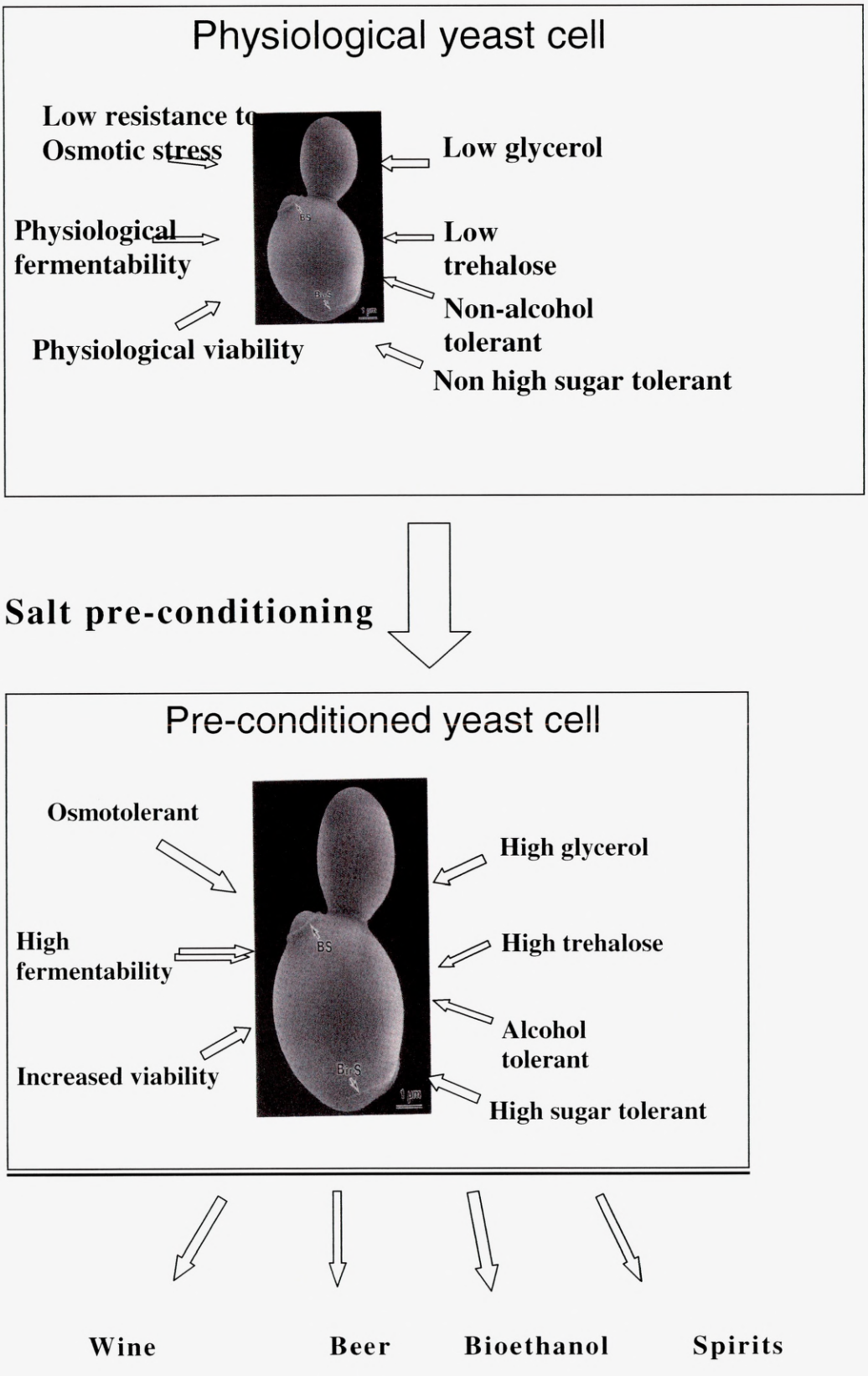

Figure 70 The effect of sodium chloride to yeast physiology

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Appendix

Appendix 1

Zbornik Matice srpske za prirodne nauke / Proc. Nat. Sci., Matica Srpska Novi Sad,
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Stelios Logothetis[1]
Graeme Walker[1]
Elias T. Nerantzis[2]*
1 University of Abertay Dundee, School of Contemporary Sciences,
Bell Street DD1 HG1 Dundee, Scotland
2 TEI of Athens Department of Oenology and Spirit Technology,
Biotechnology & Industrial Fermentations Lab Agiou Spiridonos
12210 Egaleo, Athens, Greece E-mail: enerat@teiath.gr
• Author to whom all correspondence should be addressed

EFFECT OF SALT HYPEROSMOTIC STRESS ON YEAST CELL VIABILITY

ABSTRACT: During fermentation for ethanol production, yeasts are subjected to different kinds of physico-chemical stresses such as: initially high sugar concentration and low temperature; and later, increased ethanol concentrations. Such conditions trigger a series of biological responses in an effort to maintain cell cycle progress and yeast cell viability. Regarding osmostress, many studies have been focused on transcriptional activation and gene expression in laboratory strains of *Saccharomyces cerevisiae*. The overall aim of this present work was to further our understanding of wine yeast performance during fermentations under osmotic stress conditions. Specifically, the research work focused on the evaluation of NaCl-induced stress responses of an industrial wine yeast strain *S. cerevisiae* (VIN 13), particularly with regard to yeast cell growth and viability. The hypothesis was that osmostress conditions energized specific genes to enable yeast cells to survive under stressful conditions. Experiments were designed by pretreating cells with different sodium chloride concentrations (NaCl: 4%, 6% and 10% w/v) growing in defined media containing D-glucose and evaluating the impact of this on yeast growth and viability. Subsequent fermentation cycles took place with increasing concentrations of D-glucose (20%, 30%, 40% w/v) using salt-adapted cells as inocula. We present evidence that osmostress induced by mild salt pre-treatments resulted in beneficial influences on both cell viability and fermentation performance of an industrial wine yeast strain.
KEY WORDS: *Saccharomyces cerevisiae*, wine yeast, salt stress, cell growth, cell Viability

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Appendix 2

International Wine Challenge 2008

Your award winning wines

Click on a wine name for more details, or click on 'Back to Search' to narrow down the result.

Back to Search

Medal	Wine	Style	Average Price	Buy this wine at
	Plagies Peristeras Chardonnay , Greece, 2007 Produced by Georgakopoulos Estate	White Still		Georgakopoulos Estate

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Appendix 3

International Wine Challenge 2009

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- INTERNATIONAL WINE CHALLENGE
- LIBRARY COLLECTION
- RESULTS
- IWC SHOP
- USEFUL WINE LINKS

Award winning wine in detail Back to search results

Onirovatis, 2007

Awaiting image

From: Central Greece, Greece
Made with: Malagousia

Available at
Georgakopoulos Estate | WineSearcher.com

Footer information

International Wine Challenge

- Enter the Challenge
- Rules for Entry
- Discovery Tasting
- How the Challenge Works
- Judges
- Awards Dinner
- Contact Us
- Sponsors
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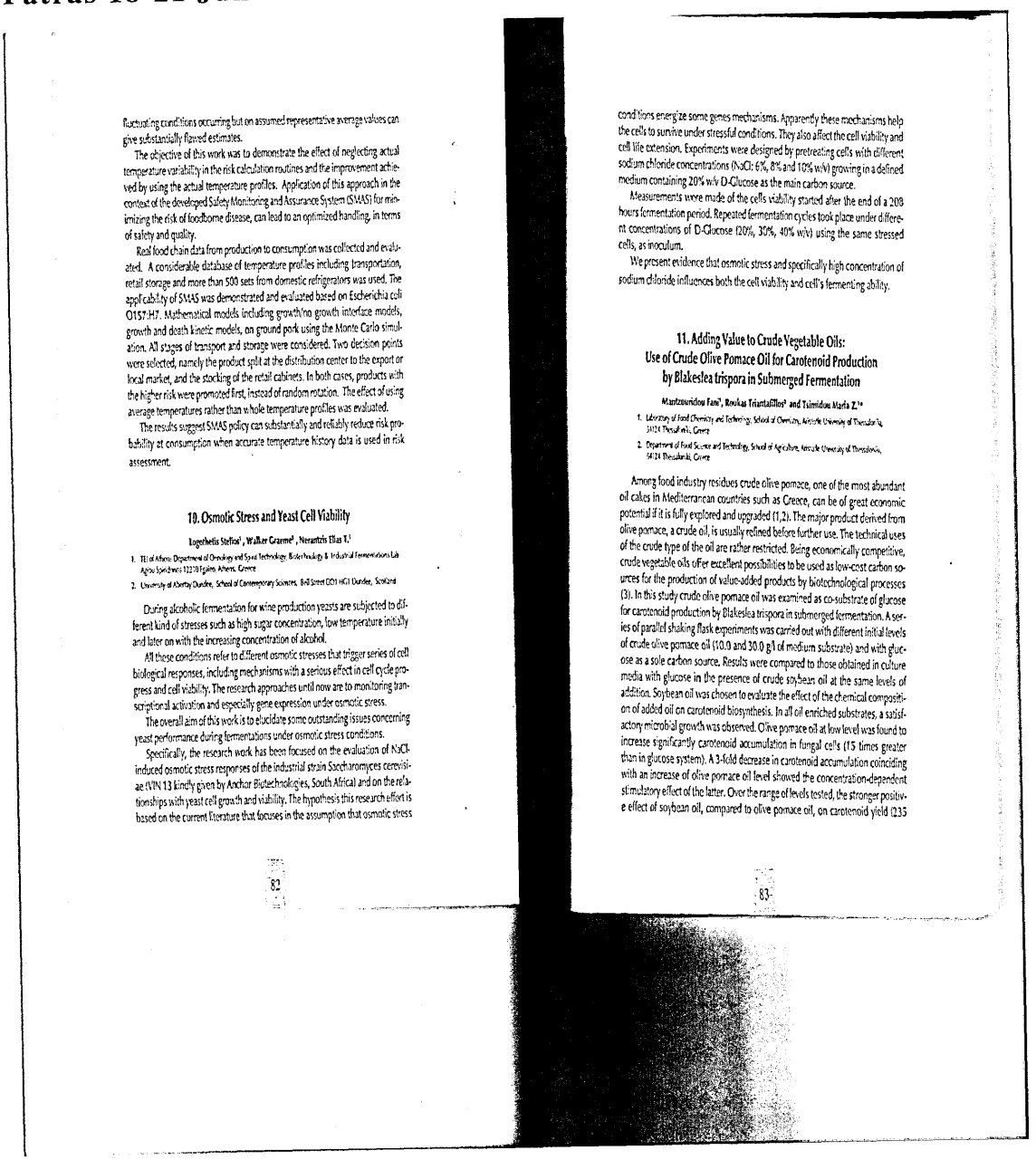
Library Collection

Appendix 4

2nd International Congress on Bioprocesses in Food Industries ICBF Patras 18-21 June 2006



fluctuating conditions occurring but on assumed representative average values can give substantially flawed estimates.

The objective of this work was to demonstrate the effect of neglecting actual temperature variability in the risk calculation routines and the improvement achieved by using the actual temperature profiles. Application of this approach in the context of the developed Safety Monitoring and Assurance System (SMAS) for minimizing the risk of foodborne disease, can lead to an optimized handling, in terms of safety and quality.

Real food chain data from production to consumption was collected and evaluated. A considerable database of temperature profiles including transportation, retail storage and more than 500 sets from domestic refrigerators was used. The applicability of SMAS was demonstrated and evaluated based on Escherichia coli O157:H7. Mathematical models including growth/no growth interface models, growth and death kinetic models, on ground pork using the Monte Carlo simulation. All stages of transport and storage were considered. Two decision points were selected, namely the product split at the distribution center to the export or local market, and the stocking of the retail cabinets. In both cases, products with the higher risk were promoted first, instead of random rotation. The effect of using average temperatures rather than whole temperature profiles was evaluated.

The results suggest SMAS policy can substantially and reliably reduce risk probability at consumption when accurate temperature history data is used in risk assessment.

10. Osmotic Stress and Yeast Cell Viability

Logothetis Stefios[1], Walker Graeme[2], Nerantzis Elias T.[1]

1. TEI of Athens, Department of Oenology and Food Technology, Biotechnology & Industrial Fermentations Lab Agiou Spyridonos 12210 Egaleo, Athens, Greece
2. University of Abertay Dundee, School of Contemporary Sciences, Bell Street DD1 HG1 Dundee, Scotland

During alcoholic fermentation for wine production yeasts are subjected to different kind of stresses such as high sugar concentration, low temperature initially and later on with the increasing concentration of alcohol.

All these conditions refer to different osmotic stresses that trigger series of cell biological responses, including mechanisms with a serious effect in cell cycle progress and cell viability. The research approaches until now are to monitoring transcriptional activation and especially gene expression under osmotic stress.

The overall aim of this work is to elucidate some outstanding issues concerning yeast performance during fermentations under osmotic stress conditions.

Specifically, the research work has been focused on the evaluation of NaCl-induced osmotic stress responses of the industrial strain Saccharomyces cerevisiae (VIN 13 kindly given by Anchor Biotechnologies, South Africa) and on the relationships with yeast cell growth and viability. The hypothesis this research effort is based on the current literature that focuses in the assumption that osmotic stress

conditions energize some genes mechanisms. Apparently these mechanisms help the cells to survive under stressful conditions. They also affect the cell viability and cell life extension. Experiments were designed by pretreating cells with different sodium chloride concentrations (NaCl: 6%, 8% and 10% w/v) growing in a defined medium containing 20% w/v D-Glucose as the main carbon source.

Measurements were made of the cells viability started after the end of a 208 hours fermentation period. Repeated fermentation cycles took place under different concentrations of D-Glucose (20%, 30%, 40% w/v) using the same stressed cells, as inoculum.

We present evidence that osmotic stress and specifically high concentration of sodium chloride influences both the cell viability and cell's fermenting ability.

11. Adding Value to Crude Vegetable Oils: Use of Crude Olive Pomace Oil for Carotenoid Production by Blakeslea trispora in Submerged Fermentation

Mantzouridou Fani[1], Roukas Triantafillos[2] and Tsimidou Maria Z.[1]*

1. Laboratory of Food Chemistry and Technology, School of Chemistry, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece
2. Department of Food Science and Technology, School of Agriculture, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece

Among food industry residues crude olive pomace, one of the most abundant oil cakes in Mediterranean countries such as Greece, can be of great economic potential if it is fully explored and upgraded (1,2). The major product derived from olive pomace, a crude oil, is usually refined before further use. The technical uses of the crude type of the oil are rather restricted. Being economically competitive, crude vegetable oils offer excellent possibilities to be used as low-cost carbon sources for the production of value-added products by biotechnological processes (3). In this study crude olive pomace oil was examined as co-substrate of glucose for carotenoid production by Blakeslea trispora in submerged fermentation. A series of parallel shaking flask experiments was carried out with different initial levels of crude olive pomace oil (10.0 and 30.0 g/l of medium substrate) and with glucose as a sole carbon source. Results were compared to those obtained in culture media with glucose in the presence of crude soybean oil at the same levels of addition. Soybean oil was chosen to evaluate the effect of the chemical composition of added oil on carotenoid biosynthesis. In all oil enriched substrates, a satisfactory microbial growth was observed. Olive pomace oil at low level was found to increase significantly carotenoid accumulation in fungal cells (15 times greater than in glucose system). A 3-fold decrease in carotenoid accumulation coinciding with an increase of olive pomace oil level showed the concentration-dependent stimulatory effect of the latter. Over the range of levels tested, the stronger positive effect of soybean oil, compared to olive pomace oil, on carotenoid yield (235

Appendix 5

From Grape 2 Wine 2nd International Junior Researchers Meeting Athens Greece 10-12 July 2008

From Grape to Wine- 2nd International Junior Researchers' Meeting
10-12 July 2008, Athens, Greece

Fermentation of high sugar concentration medium using pre-adapted yeast cells in osmotic stress using a continuous Micro-Fermentation Cassette system

St. Logothetis[1], E. Nerantzis[1], Gr. Walker[2], A. Kannelis[1], P. Tataridis[1]

[1] TEI of Athens , Department of Oenology & Beverages Technology Biotechnology and Industrial Fermentations Laboratory, Ag.Spyridona Street, Aegaleo, 12210 Athens, Greece
[2]Division of Biotechnology & Forensic Science, School of Contemporary Sciences, University of Abertay Dundee, Bell Street, Dundee DD1 1HG, Scotland, UK

Abstract

During alcoholic fermentation for wine production, yeasts are subjected to different kinds of physico-chemical stresses such as: high sugar concentration and low temperature; and secondarily, increasing ethanol concentrations. Such conditions trigger a series of biological responses in an effort to maintain cell cycle progress and yeast cell viability. Regarding osmostress, many studies have focused on transcriptional activation and gene expression in laboratory strains of *Saccharomyces cerevisiae*. The overall aim of the present work was first to further our understanding on wine yeast performance during fermentations under osmotic stress conditions and secondly to evaluate specifically, the fermentation ability of pre adapted cells on osmotic stress entrapped in calcium alginate beads and using a brand new system for continuous fermentation

The Micro-Fermentation Cassette (Nerantzis, 2006) was used using high sugar content media (30% and 50%), in a continuous mode. The MFC is the development of the WITY system (Nerantzis *et al.* 1995). It combines the versatility of the Tower fermentor of the WITY system in a smaller size cassette-type, bioreactors. It is mainly a cascade fermentation system which can carry out fermentations in multiple strain series of bioreactors which have dimensions of a cassette and is composed of two or more fermentors that they are connected with tubes and valves. The fermentation mode on this bioreactor is continuous and is achieved by a simple flow of must in the cassette. Each cassette contains a particular yeast strain pre-adapted in different osmotic stress conditions.

The biomass was hold in each individual cassette by entrapping yeast in double layer alginate beads.

Keywords: *NaCl, viability, fermentation ability, immobilization, MF Cassette*

Appendix 6

Patent Title: Method for adaptation of wine yeast cells under high concentration of NaCl

Patent Number: Greek Industrial Property Organisation: 2006 010 0269

Abstract

Method for acclimatization of wine yeast cells in conditions of high NaCl, aiming at the creation of durable cells in unfavourable conditions of culture and with high fermentative ability and viability. The production of yeasts with these attributes is particularly useful for the production of alcohol as well as for the realization of fermentations under particular conditions.